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Abstract

In multiple sclerosis (MS), demyelination results in impaired axon conduction and functional deficits. Remyelination is often observed early in the MS disease course, but over time becomes limited. Factors that may influence remyelination are important, as denuded axons have impaired neurotransmission and increased vulnerability to transection. The general belief is that remyelination requires robust oligodendrocyte progenitor (OP) amplification prior to remyelination. Myelin transcription factor 1 (Myt1) influences OP proliferation, differentiation, and myelin gene transcription *in vitro*. The potential of Myt1 to influence OP responses leading to remyelination was examined using murine hepatitis virus (MHV) induced demyelination. Myt1 expression was dramatically increased in lesioned white matter. Cells expressing Myt1 proliferated extensively during active demyelination and early remyelination, and Myt1 was observed predominantly in OPs. Increased expression of Myt1 was found within MHV lesions and in MS tissue adjacent to and within lesions. These results suggest a potential role for Myt1 in oligodendrocyte lineage cell regeneration in response to acute demyelination.

With MS being a chronic disease we were interested in the responses that occurred following chronic demyelination. Studies using the chronic cuprizone model of demyelination display limited remyelination, a depleted pool of OPs, and decreased oligodendrocytes. We now show that after chronic demyelination apoptosis continues even after cessation of cuprizone to evaluate means to promote remyelination. Overexpression of platelet-derived growth factor-A (PDGF-A) was tested with chronic cuprizone demyelination in *hPDGF-A* transgenic (tg) mice. Remyelination was

improved in *hPDGF-A* tg mice during recovery after chronic demyelination. OP density and proliferation increased only transiently in *hPDGF-A* tg mice during acute demyelination but not during chronic demyelination or recovery. Importantly, *hPDGF-A* tg mice had increased oligodendrocyte regeneration associated with reduced apoptosis during recovery. The effect of increased PDGF-A is likely as a survival factor during the regeneration of oligodendrocytes and remyelination, as preventing apoptosis of oligodendrocytes may be important not only during acute demyelination but also during chronic demyelination. Overall, we found that following demyelination Myt1 may have a potential role in the regeneration of oligodendrocyte lineage cells, whereas the overexpression of PDGF-A appears to enhance survival of newly differentiated myelinating oligodendrocytes.

The Oligodendrocyte Progenitor Response to Demyelination

by

Adam C. Vana

Thesis/dissertation submitted to the Faculty of the Neuroscience Program

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Dedication

To my wife Vanessa, who somehow always had faith in me even when I doubted myself and kept me on an even keel to finish my studies. Without her by my side I would have been lost.

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CHAPTER 1

Introduction

Background

Human disorders resulting in CNS demyelination, such as spinal cord injury, toxic insults, vascular lesions, leukoencephalopathies, and multiple sclerosis (MS), often result in focal lesions and loss of neurologic function. Traumatic spinal cord injury results in the loss of neurons but there is also a substantial loss of mature oligodendrocyte cells. Within lesions associated with spinal cord injury, mature oligodendrocytes undergo apoptosis with an ensuing loss of myelin from surviving axons (Crowe, et al., 1997). The ensuing demyelination, resulting in the impairment of action potential propagation, ultimately leads to additional neurologic disability (McTigue, et al., 2001). After demyelination in the CNS spontaneous remyelination is documented, however many of these lesions do not fully remyelinate. In fact in disorders such as MS the ability to remyelinate is often observed to decrease, or is completely lost, over time (reviewed in Prineas et al., 1993; Imitola et al., 2006).

Remyelination by mature oligodendrocytes is often found to recapitulate developmental myelination and is essential for restoring rapid conduction in previously demyelinated axons. The regulation of oligodendrocyte lineage cell responses is critical for remyelination and is likely regulated by signals within the lesion environment, such as

growth factors, cytokines and cell-cell interactions. Remyelination not only restores rapid nerve conduction but may also protect denuded axons from degeneration (reviewed in Jones and Brusa, 2003), thus preventing further neurological deficits. Currently, no potential therapeutics are available to enhance remyelination. For this reason, successful lesion repair strategies following demyelination are particularly needed to restore function and prevent further axonal loss.

Within the adult CNS neural stem/progenitor cells (NSCs) persist with the ability to generate mature neural cells (i.e. neurons, astrocytes, and oligodendrocytes). These self-renewing multipotent NSCs are found distributed in the CNS within the subventricular zone (SVZ) (Rietze et al., 2001), subgranular zone of the hippocampus (SGZ) (Yagita et al., 2002), cortex (Tamaki et al., 2002), and white matter (Gensert and Goldman, 1996; Horner and Gage, 2000). This population of endogenous NSCs may offer a valuable resource for manipulation and directed differentiation toward an oligodendrocyte lineage cell fate in the promotion of CNS remyelination and self-repair.

The progression of oligodendrocyte lineage cells from immature pre-oligodendrocyte progenitor cells, to oligodendrocyte progenitor cells (OPs), to pre-oligodendrocytes, and finally to mature myelinating oligodendrocytes is well characterized (Armstrong, 1998). OP responses during myelination and remyelination are similar with proliferative amplification, migration, terminal differentiation, and survival of newly differentiated oligodendrocytes. A variety of growth factors, such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), are known to modulate proliferation and differentiation of OPs *in vitro* and *in vivo* (McKinnon et al., 1990; Wolswijk and Noble, 1992; Frost et al., 2003; Murtie et al., 2005). Growth factor

regulation of OP responses occurs by acting through intracellular signaling cascades, which influences transcriptional molecules and finally gene expression. Within the lesions of MS patients, OPs and pre-oligodendrocytes are frequently present (Wolswijk, 1997, 1998, 2000; Chang et al., 2000, 2002; Maeda et al., 2001; Wilson et al., 2006). Mature oligodendrocytes may also be present in lesions, however these oligodendrocytes generally do not produce myelin, and thus provide minimal contribution toward remyelination after demyelination (Chang et al., 2002). While the pool of OPs often are quiescent and do not differentiate into mature oligodendrocytes (Wolswijk, 1997, 1998; Chang et al., 2002; Wilson et al., 2006), it is believed that the mature oligodendrocytes responsible for remyelination after demyelination are derived from the OP pool. The generally accepted dogma of remyelination is that the proliferation of OPs precedes oligodendrocyte regeneration and remyelination (Carroll et al., 1998; Keirstead et al., 1998; Redwine and Armstrong, 1998; Reynolds et al., 2001; Watanabe et al., 2002). These processes are known to be influenced by transcription factors and growth factors. The proliferation of OPs is robust in acute demyelination, but is limited or nonexistent in chronic demyelination, as OPs are either depleted or quiescent. Early in the disease course of MS remyelination is extensive with a restoration of function, but over time remyelination after multiple relapses is limited or completely absent. Animal models of demyelination offer the necessary means to study the molecular/cellular regulation of remyelination by transcription factors and growth factors after acute and chronic demyelination.

Models of demyelination

There are numerous models of experimental demyelination with remyelination, including experimental allergic encephalomyelitis (EAE; in some instances referred to as experimental autoimmune encephalomyelitis), viral models, and neurotoxicants. These experimental models are important for testing the function of strategies that may positively influence remyelination for diseases such as MS. Within our lab we have extensively studied two complementary models of demyelination with remyelination: infection with murine hepatitis virus strain A59 (MHV-A59) and ingestion of the neurotoxicant cuprizone.

Murine hepatitis virus strain A59

The MHV-A59 model of demyelination with spontaneous remyelination has been well characterized (reviewed in Matthews et al., 2002a). Intracranial injection of MHV-A59, a member of the coronavirus family, results in infection of white matter cells, CNS demyelination followed by extensive remyelination, viral clearance, and recovery of motor function (Jordan et al., 1989; Redwine and Armstrong, 1998). Within the first week post-injection demyelination and oligodendrocyte loss is observed in the spinal cord and by the second week myelin degeneration is extensive (Jordan et al., 1989). Approximately four week post-injection corresponds with the onset of remyelination, and by eight week post-injection remyelination is extensive with associated recovery of function (Armstrong et al., 1990; Redwine and Armstrong, 1998). The complexity of the MHV-A59 model is similar to MS with multifocal lesions, blood brain barrier breakdown (BBB), and lymphocytic infiltration along with expression of cytokines, chemokines and cell adhesion molecules (Matthews et al., 2002b).

Cuprizone

The cuprizone model of demyelination is an excellent complement to the MHV-A59 model. The susceptibility of oligodendrocyte cells to cuprizone toxicity is likely a facet of the high metabolic demands that are required to maintain the myelin sheath (Komoly et al., 1987; Fujita et al., 1990; Morell et al., 1998; Matsushima and Morell, 2001), as cuprizone is a copper chelator believed to negatively influence mitochondrial function. This model offers a far less complex lesion environment than many of the other experimental models of demyelination with remyelination, and thus limiting possible variables allows a more finite assessment of the specific factors involved in the remyelination process.

A fundamental advantage of the cuprizone model is the spatial and temporal reproducibility of lesions specifically within the corpus callosum. Within the cuprizone model the BBB remains intact (Kondo et al., 1987; Bakker and Ludwin, 1987; Pedchenko and Levine, 1999; McMahon et al., 2001, 2002), with minimal immune cell infiltration (McMahon et al., 2002). The acute cuprizone model offers a highly reproducible time course of demyelination with remyelination, which is well-characterized over a nine week time period (mice on cuprizone for six weeks followed by three weeks of recovery). Maximal oligodendrocyte loss is apparent at three weeks, while at six weeks (the time point when acute cuprizone treatment is stopped) maximal myelin loss is observed. During a three week recovery period following cuprizone ingestion there is a substantial repopulation of oligodendrocytes and remyelination of the corpus callosum. However, when mice are maintained on cuprizone for twelve weeks (chronic cuprizone model of

demyelination) the pool of OPs becomes depleted, oligodendrocyte regeneration is compromised, and remyelination is limited (Mason et al., 2001, 2004; Armstrong et al., 2006). Even when allowing for up to six weeks for recovery after the cessation of chronic cuprizone treatment, the OP pool remains depleted, oligodendrocyte density remains severely decreased, and remyelination is limited (Armstrong et al., 2006). The acute and chronic cuprizone induced models of demyelination offer the ability to examine a disease course similar to the early and late phases of those observed in MS.

Transcription Factors

Transcription factors bind to specific DNA sequences as well as to the RNA polymerase complex and other cofactors, which results in activation or repression of gene transcription. Growth factors and other molecules, such as cytokines, are known to ultimately regulate the production of transcription factors. Myelin transcription factor 1 (Myt1), a tissue-specific transcription factor, has been implicated in the differentiation of oligodendrocytes (Armstrong et al., 1995; Nielsen et al., 2004) and neuronal cells (Bellefroid et al., 1996). Myt1 was originally cloned based upon its affinity for binding a site within the promoter of the proteolipid protein (PLP) gene, the most abundantly transcribed myelin gene (Kim and Hudson, 1992). Classified as a zinc-finger DNA-binding protein, Myt1 is expressed in neural precursors and immature oligodendrocyte lineage cells (Armstrong et al., 1995; Bellefroid et al., 1996; Kim et al., 1997). Structurally, Myt1 contains seven zinc-fingers (clustered into three N-terminal zinc-fingers and four C-terminal zinc-fingers, with both sets of fingers able to bind independently to the PLP promoter), as well as a putative transcriptional activation

domain and alpha-helical protein-protein interaction domain (Kim and Hudson, 1992; Matsushita et al., 2002). In pathological conditions of the CNS, Myt1 upregulation has been observed in spinal cord injury (Wrathall et al., 1998), gliomas (Armstrong et al., 1997), and MS cases (unpublished data, R.C. Armstrong). Determining how Myt1 regulates neural stem/progenitor cells with the ability to generate oligodendrocyte lineage cells may ultimately lead to the development of therapies to induce remyelination after pathology without the consequence of possible tumor formation.

Recent work investigated the function of Myt1 *in vitro* in oligodendrocyte lineage cells (Nielsen et al., 2004). A replication-incompetent retrovirus expressing the four zinc-finger DNA binding domain of Myt1 (4FMyt1) was utilized. The 4FMyt1 contains the C-terminal four-finger DNA binding element, but lacks the putative acidic transcriptional activation domain and the alpha-helical protein-protein interaction domain. The expectation is that 4FMyt1 competes with endogenous Myt1 for binding of DNA promoter/enhancer elements and thus act in a manner consistent with that of a dominant-negative by interfering with the typical function of endogenous Myt1. The overexpression of 4FMyt1 *in vitro* resulted in a significant decrease in proliferation of OPs exposed to medium with the mitogens PDGF and FGF. While the overexpression of 4FMyt1 decreased OP differentiation into oligodendrocytes in response to differentiation medium with high insulin and thyroid hormone. The 4FMyt1 had no effect upon either astrocytes, which do not express endogenous Myt1, or mature oligodendrocytes that were infected as OPs, which are post-mitotic and do not express Myt1. A putative target of Myt1 in oligodendrocyte lineage cells is the myelin gene promoter. The myelin gene 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) is expressed by oligodendrocyte

lineage cells and cotransfection of full-length Myt1 into rat CG4 oligodendrocyte progenitor cell line along with a CNP-luciferase reporter resulted in activation of the CNPase promoter (Nielsen et al., 2004). These *in vitro* results strongly implicate a role for Myt1 in proliferation and differentiation of oligodendrocyte lineage cells and myelin gene expression.

Growth Factors

The proliferation, migration, differentiation, and survival of oligodendrocyte lineage cells are regulated by complex interactions of multiple signals. Growth factors have profound influences on oligodendrocyte lineage cell responses. Among growth factors, strong evidence exists that PDGF may be a critical regulator of oligodendrocyte generation during developmental myelination, as well as in the regeneration of oligodendrocytes during remyelination.

Role of PDGF in the CNS

The PDGF family currently consists of four ligands, PDGF-A, B, C, and D, and two high affinity receptors, PDGFαR and PDGFβR (reviewed in Shih and Holland, 2006). PDGF signaling is complex, as the ligand PDGF polypeptide chains cross-link via disulfide bonds to form homodimers or heterodimers. These PDGF ligand dimers then bind to two PDGFRs simultaneously to yield a dimerized receptor. The binding properties of PDGFRs with the four ligand family members are still not fully characterized. However, the general consensus is that PDGFαR can bind PDGF-A,

PDGF-B, or PDGF-C, while the PDGFβR can bind PDGF-B or PDGF-D (reviewed in Shih and Holland, 2006). The PDGFRs are members of the receptor tyrosine kinase (RTK) family of receptors and activate classical RTK signal transduction pathways including: phospatidylinositol 3-kinase (PI3K), Ras-Mitogen Activated Protein Kinase (MAPK), Src family kinase, Stat transcription factors, and phospholipase Cγ (PLC-γ) pathways (reviewed in Shih and Holland, 2006). However, while *in vivo* studies demonstrate considerable overlap in signal transduction between PDGFαR and PDGFβR, they also demonstrate the distinct functions of each receptor (Klinghoffer et al., 2001). Within the CNS the only PDGFR expressed on OPs is the PDGFαR (Wolswijk and Noble, 1992, Shi et al., 1998), thus homodimerization of PDGFαR induction is the manner in which signal transduction occurs in oligodendrocyte lineage cells. OP cells within the CNS are PDGFαR positive, and in demyelinated lesions these PDGFαR positive cells are highly proliferative, as indicated by incorporation of the thymidine analog bromodeoxyuridine (BrdU; Redwine and Armstrong, 1998). Following acute demyelination an expansion of the pool of PDGFαR positive OPs occurs along with an increase in astrocyte derived PDGF-A, which precedes remyelination and a restoration of function in mice (Redwine and Armstrong, 1998).

PDGF-A

PDGF-A is expressed by neurons and astrocytes throughout the CNS (Richardson et al., 1988; Pringle et al., 1992; Yeh et al., 1993; Maxwell et al., 1998), with PDGF-A acting as a potent mitogen of OPs *in vitro* and *in vivo* (Richardson et al., 1988,

Barres et al., 1993; Calver et al., 1998; Fruttiger et al., 1999). An increased expression of PDGF-A in astrocytes has been observed in demyelinated lesions of the CNS (Redwine and Armstrong, 1998; Hinks and Franklin, 1999, 2000). Increased OP cell density is directly proportional to the amount of PDGF-A expression in a non-saturable manner, even in instances of more than a ten-fold increase, indicating that PDGF is the limiting factor determining OP cell number during development (van Heyningen et al., 2001). PDGF-A also facilitates motility of OPs *in vitro* (Armstrong et al., 1990), and would likely result in PDGF-A production from astrocytes within lesions to induce OPs to migrate into demyelinated lesions. Also, PDGF-A acts as a survival factor *in vitro* and *in vivo* for newly differentiated oligodendrocytes (Barres et al., 1992). Thus, within a lesion the increased production of PDGF-A from astrocytes may lead to more oligodendrocytes surviving to become myelinating oligodendrocytes. Transgenic and knock-out PDGF-A and PDGFαR mice have been developed and allow us models to study PDGF effects on remyelination.

In *PDGFαR* knockout mice the homozygous null *PDGFαR* mutation is embyonic lethal and mice generally die between E14 and E16 (Soriano, 1997). This makes it impossible to do studies on remyelination with these mice. However, *PDGFαR* heterozygous mice have been examined, as *PDGFαR* heterozygous mice have a normal lifespan and express half the normal amount of PDGFαR (Soriano, 1997). Following acute cuprizone induced demyelination and allowing for recovery, the *PDGFαR* heterozygous mice have a decreased density of OPs and oligodendrocytes when compared

with wild-type littermates (Murtie et al., 2005). However, even with a significantly lower density of OPs, and a subsequently lower density of oligodendrocytes, spontaneous remyelination following acute cuprizone treatment was similar between *PDGFαR* genotypes (Murtie et al., 2005). In *PDGFαR* heterozygous mice although there was a limited OP response and oligodendrocyte regeneration, remyelination was extensive indicating that a robust OP response after acute demyelination is not necessary for remyelination.

The overexpression of PDGF-A was analyzed in transgenic mice with the PDGF-A gene inserted and under the control of a cell specific promoters. These studies were done in mice with mouse PDGF-A under the control of the neuron-specific enolase promoter (*NSE-mPDGF-A*) and with human PDGF-A under the control of the astrocyte-specific GFAP promoter (*GFAP-hPDGF-A*) (Fruttiger et al., 2000). The overexpression of the short, freely diffusible splice isoforms of PDGF-A from neurons or astrocytes resulted in an increased density of OP cells (Calver et al, 1998; Fruttiger et al., 2000). *NSE-mPDGF-A* tg mice that overexpressed PDGF-A also displayed a transiently increased density of oligodendrocytes in the spinal cord during development (Calver et al., 1998). The overexpression of PDGF-A in *GFAP-hPDGF-A* transgenic mice resulted in a significant increase in the density of OPs in the corpus callosum during acute cuprizone treatment induced demyelination and in the spinal cord dorsal column after lysolethicin injection (Woodruff et al., 2004). However, the analysis of oligodendrocyte density and remyelination in *hPDGF-A* transgenic mice after demyelination was not analyzed fully in the Woodruff study. It is likely that in the Woodruff et al. (2004) study that they would

not have seen a difference in remyelination, as the response after acute demyelination is so robust that differences between wild type and transgenic mice would likely be negligible.

Significance

In these studies we were interested in the OP response to acute and chronic demyelination. The generally accepted paradigm has been that following demyelination the proliferation of OPs generally precedes oligodendrocyte regeneration and remyelination. Proliferation of OPs in acute demyelination preceded oligodendrocyte regeneration, however in chronic demyelination an OP response was nonexistent. We characterized the cell phenotypes that expressed the transcription factor Myt1 in the normal and acutely demyelinated CNS. Myt1 expression was initially observed in neural stem cells, which then proceed toward an oligodendrocyte lineage cell fate. Myt1 expression was observed in the majority of OPs, as well as a small number of terminally differentiated oligodendrocytes which expressed Myt1 for a short time prior to myelin gene transcription, similar to developmental myelination. The density of Myt1 cells increased during acute demyelination, specifically when OPs are known to proliferate, and these Myt1 positive cells were intimately involved in the regeneration of new oligodendrocytes, which then went on to remyelinate and restore function. The overexpression of PDGF-A during chronic demyelination resulted in the survival of newly differentiated oligodendrocytes. These newly generated oligodendrocytes were derived from a depleted pool of OPs, as the overexpression of PDGF-A resulted in a larger population of these newly derived oligodendrocytes surviving, thus enhancing

remyelination following chronic demyelination. Currently a dearth of therapeutics exist with the potential to promote remyelination, and a further understanding of the specific molecules and cells involved in myelination/remyelination may yield these much sought after therapeutics for such debilitating diseases as MS.

CHAPTER 2

Myelin Transcription Factor 1 (Myt1) Expression in Demyelinated Lesions of Rodent and Human CNS

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ABSTRACT

Myelin transcription factor 1 (Myt1) influences developing oligodendrocyte progenitor (OP) proliferation, differentiation, and myelin gene transcription *in vitro*. The potential of Myt1 to influence OP responses leading to remyelination was examined using murine hepatitis virus strain A59 to induce spinal cord demyelination. The density of Myt1 expressing cells was dramatically increased in lesioned white matter. Myt1 expressing cells proliferated most extensively during active demyelination followed by maximal accumulation during early remyelination. Cells with nuclear Myt1 immunoreactivity were mainly OPs, with additional phenotypes being either oligodendrocytes or neural stem cells. Mice with corpus callosum demyelination induced by cuprizone treatment also demonstrated an increase of Myt1 expressing cells in lesions. Furthermore, multiple sclerosis lesions demonstrated increased Myt1 expression in both the periplaque white matter adjacent to lesions and within early remyelinating lesions. These results suggest a potential role for Myt1 in the regeneration of oligodendrocyte lineage cells in response to demyelination.

INTRODUCTION

Demyelination and oligodendrocyte loss in the CNS can result from multiple sclerosis (MS), spinal cord injury, toxic insults, and neurodegenerative disorders. Functionally, oligodendrocytes produce the myelin sheath which enwraps axons to facilitate neurotransmission. Demyelination leads to slowing or blockade of action potential conduction. In addition, without the protective effect of myelin, denuded axons are more vulnerable to transection which can lead to permanent neurologic impairment (Ferguson et al., 1997; Trapp et al., 1998).

Spontaneous remyelination has been observed in MS lesions following initial episodes of acute demyelination, yet the capacity for remyelination becomes limited with subsequent prolonged or repeated episodes of demyelination (Prineas et al., 1993; Raine and Wu, 1993; Bruck et al., 2003). Animal models of experimental demyelination have shown that remyelination requires oligodendrocyte progenitor (OP) cells in the proximity of demyelinated lesions to proliferate and then differentiate into mature oligodendrocytes which can then myelinate denuded axons (Reynolds et al., 2001; Watanabe et al., 2002). This sequence of cellular responses must be orchestrated through transcriptional control of the complex gene expression patterns that regulate OP proliferation, differentiation, and eventual synthesis of myelin components.

Several previous studies implicate myelin transcription factor 1 (Myt1) as potentially important in regulating OP responses in the developing and adult CNS. Myt1 is a Cys-Cys-His-Cys (CCHC) zinc-finger DNA binding protein that was originally identified based upon binding affinity within the promoter region of the proteolipid (PLP) gene, the most abundantly transcribed CNS myelin gene (Kim and Hudson, 1992).

During development of the oligodendrocyte lineage, Myt1 is localized within nuclei of immature cells and then down-regulated after terminal differentiation and accumulation of myelin proteins in mature oligodendrocytes (Armstrong et al., 1995). Myt1 continues to be expressed in germinal zones of the adult CNS and is upregulated in gliomas (Armstrong et al., 1997) and following spinal cord traumatic injury (Wrathall et al., 1998). *In vitro*, expression of a dominant negative form of Myt1 showed that Myt1 can regulate a critical transition in oligodendrocyte lineage cell development by modulating OP proliferation relative to terminal differentiation and upregulation of myelin gene transcription (Nielsen et al., 2004).

To examine the potential role of Myt1 in regulating the OP responses required for remyelination, the current study determines the expression of Myt1 relative to the proliferative status and phenotype of cells in white matter during the progression of experimental demyelination followed by spontaneous remyelination. Murine hepatitis virus (MHV) strain A59 infection was used to induce focal demyelinated lesions of spinal cord white matter from lytic infection of oligodendrocytes with subsequent lymphocytic infiltration and virus clearance (Redwine and Armstrong, 1998; Matthews et al., 2002). Studies in the MHV model were complemented by additional analysis using cuprizone demyelination of the corpus callosum, which does not involve a strong lymphocytic response or breakdown of the blood-brain barrier (Ludwin, 1980; Matsushima and Morell, 2001). Finally, MS lesions were examined for Myt1 immunoreactivity relative to stage of demyelinating activity.

MATERIALS AND METHODS

Animals

C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were maintained in the Uniformed Services University of the Health Sciences (USUHS) animal housing facility in accordance with guidelines of the National Institutes of Health and the USUHS Institutional Animal Care and Use Committee.

Murine Hepatitis Virus (MHV) Model of Spinal Cord Demyelination

Intracranial infection with MHV coronavirus strain A59 in C57Bl/6 mice results in focal areas of demyelination throughout the spinal cord, with a complex pathology that includes macrophage/microglial activation, astrogliosis, and lymphocyte infiltration (Matthews et al., 2002). As previously described (Redwine and Armstrong, 1998; Armstrong et al., 2005), 4 week old female C57Bl/6 mice were anesthetized and injected intracranially with MHV strain A59 diluted in sterile PBS to 1000 plaque forming units (PFU) per 10 μl injection volume. Control animals were injected with 10 μl of sterile PBS.

The hang time test was used to assess motor dysfunction in mice with MHV spinal cord demyelinated lesions (Redwine and Armstrong, 1998; Frost et al., 2003; Armstrong et al., 2005). Mice were placed on a cage top which was then inverted. The time was recorded for the duration each mouse could hang from the cage top, up to a maximum of 60 seconds. For each disease time point, hang time tests were performed in triplicate with 10 to 20 seconds between trials. Hang times were recorded on days 0, 7, 10, 14, 17, 21, 24, 28 and then every 7 days for up to 8 weeks post infection (wpi). This study used only mice with a pattern of severe disability followed by spontaneous

recovery, as previously characterized for this demyelinating disease model (Redwine and Armstrong, 1998; Frost et al., 2003). In addition to the hang time assessments, on the same days a clinical scores was assigned as follows: 0 for no evidence of paresis/paralysis, 1-5 for paresis/paralysis in one to five appendages, and 6 for morbidity (Redwine and Armstrong, 1998; Armstrong et al., 2005).

Cuprizone Model of Experimental Demyelination

Cuprizone ingestion results in a reproducible pattern of extensive corpus callosum demyelination by 5-6 weeks which, upon return to normal chow, is followed by spontaneous remyelination (Matsushima and Morell, 2001; Armstrong et al., 2002). Male C57Bl/6 mice at 8 weeks of age were started on 0.2% (w/w) cuprizone diet (finely powdered oxalic bis(cyclohexylidenehydrazide); Aldrich, Milwaukee, WI) mixed into milled chow (Harland Teklad, Certified LM-485 code 7012CM).

Preparation of Mouse Tissue Sections

At 2, 4, and 8 wpi, mice were intracardially perfused with 4% paraformaldehyde (Sigma, St. Louis, MO). Spinal cords were dissected out of the vertebral column and post-fixed in 4% paraformaldehyde at 4^{o}C overnight, cryoprotected in 30% sucrose (Sigma, St. Louis, MO) at 4^{o}C overnight, embedded in Tissue Tek OCT (Sakura, Torrance, CA) and stored at –80^{o}C. Spinal cord segments were sectioned at 15 μm on a cryostat (Bright Instruments, England), and thaw mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA).

***In situ* Hybridization of Mouse Tissue Sections**

In situ hybridization and preparation of digoxigenin-labeled riboprobes were performed with modifications of methods previously detailed (Redwine and Armstrong,

1998; Messersmith et al., 2000). The antisense ribonucleotide probe to detect myelin transcription factor 1 (Myt1) mRNA transcripts was synthesized from a cDNA template (gift from Dr. Lynn Hudson; National Institutes of Health; Kim et al., 1997) by *in vitro* transcription (MAXIscript SP6; Ambion, Austin, TX) to be complementary to a 1.2 kilobase mRNA fragment encoding the 3' untranslated region of mouse Myt1 (Kim et al., 1997). Spinal cord cryosections were washed in PBS, treated with 20 U/ml Proteinase K (Sigma, St. Louis, MO) at 37^{o}C for 40 minutes, and then hybridized with 400 ng of riboprobe per ml at 53^{o}C overnight. Digoxigenin was detected with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche, Indianapolis, IN), followed by reaction with NBT/BCIP substrate overnight (DAKO, Carpinteria, CA).

BrdU Incorporation and Detection in Mice

At 4 hours and 2 hours prior to perfusion, mice were injected intraperitoneally with 200 mg/kg bromodeoxyuridine (BrdU) (Sigma, St. Louis, MO). Following *in situ* hybridization detection of Myt1 transcripts, sections were processed for immunostaining with a monoclonal anti-BrdU antibody directly conjugated with horseradish peroxidase (mouse monoclonal IgG Fab fragment; Roche, Indianapolis, IN). Peroxidase activity was detected by incubation with 3,3'-diaminobenzidine (DAB; Vector Labs, Burlingame, CA).

Immunohistochemistry of Mouse Tissue Sections

To detect Myt1, mouse spinal cord tissue sections were immunostained using the αMyt1-His rabbit polyclonal antibody (1:100; gift from Dr. Lynn Hudson; National Institutes of Health), which has been characterized in a previous study (Armstrong et al., 1995). OP cells were identified with a rat monoclonal anti-PDGFαR antibody (APA5

diluted 1:200; BD Biosciences Pharmingen, San Diego, CA) and mature oligodendrocytes were labeled with a mouse monoclonal antibody against CC1 (1:20; Oncogene Research Products, Cambridge, MA). Neural stem cells were identified with a biotinylated rat anti-mouse monoclonal antibody against Musashi1 (1:2000; gift from Dr. Hideyuki Okano; Keio University School of Medicine, Tokyo, Japan; Kaneko et al., 2000). Astrocytes were identified with a mouse monoclonal antibody against glial fibrillary acidic protein (GFAP; 1:20; Roche, Indianapolis, IN). Primary antibodies used to identify immune cells were rat anti-mouse monoclonal pan T-cell antibody (Ox-52 diluted 1:1000; BD Biosciences Pharmingen, San Diego, CA), rat anti-mouse FITC conjugated monoclonal B-cell antibody (CD45R diluted 1:25; BD Biosciences Pharmingen, San Diego, CA), and rat monoclonal macrophage/microglia antibody (Mac1 diluted1:100; Roche, Indianapolis, IN).

All secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA). Myt1 was detected with Cy3 donkey anti-rabbit IgG. PDGFαR, Ox52, and Mac1 were detected with biotinylated donkey anti-rat IgG followed by fluorescein tyramide signal amplification (Perkin Elmer Life Sciences, Boston, MA). Musashi1 was detected by fluorescein tyramide signal amplification. CC1 and GFAP were detected with FITC donkey anti-mouse IgG**.** Sections were stained with DAPI (Sigma, St. Louis, MO) to detect nuclei.

Quantitative Analysis in Mouse Tissue Sections

For cell density quantification, areas within transverse spinal cord sections were measured using Spot2 software and all labeled cells within each area was counted with a 40x objective to determine cells per mm^2. For each condition and time point examined,

at least three mice were analyzed with at least three sections included for each mouse. Unpaired Student's *t*-tests were used to distinguish significant differences between groups. For hang time assessment, differences between PBS-injected controls and MHV-injected animals were analyzed with a one-way ANOVA with repeated measures followed by Tukey's post hoc tests.

Multiple Sclerosis and Control Tissues

The pathological analysis of MS autopsy and biopsy samples was approved by the Mayo Clinic Institutional Review Board (IRB #2067-99). Eight cases were selected from a larger series of MS cases with detailed clinical follow up (n = 130) derived from the MS Lesion Project cohort (Lucchinetti; NMSS grant RG 3185-A2; B3). Control tissue consisted of adult human brain subcortical white matter areas chosen from autopsy cases with no known neurological involvement. Two autopsy control cases were obtained from the Brain and Tissue Bank for Developmental Disorders (Baltimore, MD) and one was obtained from the Mayo Clinic.

Histopathology and Staging of Demyelinating Activity

Specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Slices 4 μm thick were stained with haematoxylin and eosin (HE), Luxol-fast blue (LFB) and periodic acid-Schiff (PAS) as well as Bielschowsky´s silver impregnation. Immunohistochemical staining was performed with a biotin-avidin or an alkaline phosphatase/anti-alkaline phosphatase technique. The primary antibodies used were anti-myelin basic protein (MBP, Boehringer Mannheim, Germany), anti-CD3 (T cells, Dako, Denmark), anti-CD8 (cytotoxic T cells, Dako, Denmark), anti-CD20 (B cells, Dako, Denmark), anti-KiM1P (macrophages/microglial cells, Dr. Radzun, University of

Göttingen, Germany), and anti-MRP 14 (early activated macrophages, BMA Biomedicals, Switzerland). The *demyelinating activity* was classified according to the presence of minor myelin proteins (MOG, CNPase, MAG) in macrophages (early active lesions), the presence of early remyelination, and the absence of both active demyelination and remyelination (inactive demyelinated)(Brück et al., 1995). As white matter lesions might show more than one of these features, a total number of 8 different lesion areas were investigated (3 early active; 3 inactive; and 2 remyelinated).

Myt1 Immunohistochemistry in Human Tissues

Paraffin tissue sections were dewaxed and treated with 0.3% H_2O_2 in methanol for 30 min to minimize potential endogenous peroxidase activity. Immunostaining using the αMyt1-His rabbit polyclonal antibody (1:50; see above) was detected with horseradish peroxidase using the Elite ABC kit (Vector Labs; Burlingame, CA) with DAB as the substrate. The specificity of αMyt1-His immunoreactivity in human tissues was previously characterized by pre-absorption with excess antigen (Armstrong et al., 1997). As a further demonstration of specificity of the immunostaining reaction, selected tissues were similarly immunostained with the αMyt1L-His antibody that recognizes Myt1L, a Myt1 family member which is expressed in neuronal populations but not in oligodendrocyte lineage cells (Kim et al., 1998). Sections were counterstained with eosin.

***In situ* Hybridization in Human Tissues**

As previously described (Wong et al., 2000), paraffin sections were dewaxed and processed for *in situ* hybridization with digoxigenin labeled riboprobes to detect mRNA transcripts. Antisense riboprobes to detect mRNA transcripts for human Myt1 (Wrathall

et al., 1998) and for proteolipid protein (PLP; Hudson et al., 1987) were generated from plasmids provided by Dr. Lynn Hudson (National Institutes of Health, Bethesda, MD). For combining detection of PLP mRNA and Myt1 protein, sections were processed for PLP mRNA *in situ* hybridization sections followed by immunostaining for Myt1, as described above, without eosin counterstaining.

Human Tissue Analysis

All human tissue sections were analyzed qualitatively and tissues immunostained for Myt1 that exhibited specific and consist immunoreactivity were also assessed quantitatively. Cells with nuclear immunoreactivity for Myt1 were counted using a 100x oil objective and 10 fields were sampled within each tissue area (*i.e.* active, remyelinated, or inactive lesion, adjacent periplaque white matter - PPWM, normal white matter). Characteristics of cell morphology and anatomic localization were used to exclude cells that were expected to be distinct from oligodendrocyte lineage cells, such as lymphocytes, based on comparisons with immunostaining for myelin proteins.

Imaging of Mouse and Human Tissue Sections

All images were acquired with a Spot 2 digital camera mounted to an IX-70 microscope (Olympus, Melville, NY). For fluorescence analysis, filter sets (Chroma Technologies, Brattleboro, VT) were designed as narrow band pass sets for each channel (Cy3, FITC, and AMCA) or as a triple band pass filter set for simultaneous imaging to facilitate analysis of co-localization. Images were prepared as panels using Adobe Photoshop (Mountain View, CA).

RESULTS

Myt1 expressing cells proliferate in MHV lesions during demyelination and early remyelination.

MHV infection in C57Bl/6 mice induces focal areas of demyelination throughout the spinal cord, with subsequent spontaneous remyelination (Jordan et al., 1989; Redwine and Armstrong, 1998). To ensure reproducible MHV disease progression and severity, each mouse was tested using a well characterized hang time assay (Figure 1). The hang time readily quantifies motor impairment and recovery of function, and corresponds with histopathological findings of demyelination and remyelination (Redwine and Armstrong, 1998; Messersmith et al., 2000; Frost et al., 2003). Specifically, MHV infected mice become unable to grasp the cage top bars and hold on while hanging upside down so that the hang times are decreased significantly during the demyelination that predominates through approximately 2 weeks post infection (wpi). Spontaneous remyelination progresses and corresponds with improved hang times by 4 wpi. By 8 wpi, MHV infected mice exhibited complete recovery with hang time values equivalent to those of PBS injected controls. MHV infected mice used in this study exhibited this hang time pattern, which is characteristic of severely affected mice. Each mouse also had a clinical score of 2 or greater, indicating impaired limb movement or strength.

To determine whether Myt1 expression correlated with a specific stage of disease progression, cellular expression of Myt1 transcripts was detected by *in situ* hybridization in transverse spinal cord sections (Figure 2). In MHV infected mice, the density of Myt1 expressing cells in the white matter was increased during the demyelination phase examined at 2 wpi, as compared with PBS injected controls. This Myt1 expression

peaked during the active remyelination phase, examined at 4 wpi in the MHV infected mice. At 8 wpi, corresponding with advanced remyelination in MHV infected mice, the density of cells expressing Myt1 has declined to near that of PBS injected controls (Figure 2A).

In models of experimental demyelination, proliferation of OP cells precedes the generation of new oligodendrocytes and remyelination (Reynolds et al., 2001; Watanabe et al., 2002). To evaluate a potential role of Myt1 in proliferation in response to demyelination, *in situ* hybridization for Myt1 was combined with incorporation of bromodeoxyuridine (BrdU) (Figure 2). Mice were administered BrdU during the final 4 hours before sacrifice, which allowed detection of a sufficient proportion of cells undergoing DNA synthesis while minimizing the potential for differentiation subsequent to BrdU incorporation. The density of cells double labeled for Myt1 mRNA combined with BrdU immunohistochemistry is significantly increased during the active demyelination phase at 2 wpi (Figure 2B). By 4 wpi, the density of Myt1 and BrdU double labeled cells declined slightly yet remained significantly increased over PBS injected controls (Figures 2B). Incorporation of BrdU into cells without detectable Myt1 mRNA transcripts is also significantly increased at 2 and 4 wpi, as compared with PBS injected controls (Figure 2B), and may correspond with proliferation of astrocytes in MHV lesions (see below; Redwine and Armstrong, 1998). By 8 wpi, BrdU incorporation in sections from MHV infected mice has declined to the levels observed in sections from PBS injected control mice (Figure 2B).

Amplification of Myt1 expressing cells is specifically associated with lesion areas (Figure 3). Lesions in spinal cord white matter were identified using dark field and phase

contrast microscopy to image areas of myelin loss and vacuolation (not shown; Redwine and Armstrong, 1998; Armstrong, 2000). The overall density of cells expressing Myt1 mRNA is greatly increased within MHV lesions. In contrast, normal appearing white matter (NAWM) of MHV infected mice had a relatively low density of cells expressing Myt1, which was similar to values from the white matter of PBS injected controls.

Myt1 is localized to the nuclei of immature cell types in MHV lesions.

To determine in which cell types Myt1 may have a functional role in MHV lesions, immunostaining for cell type-specific antigens was used to characterize cell types with nuclear immunoreactivity for Myt1 in spinal cord sections from MHV lesioned mice at 4 wpi and PBS control mice (Armstrong et al., 1995). The above results correlating Myt1 expression with a proliferative population of cells in MHV lesions indicated that immature cell types may be correlated with Myt1 expression in MHV lesions. Indeed, in sections from both PBS and MHV mice, the majority of OP cells (Figure 4) identified by immunolabeling for platelet-derived growth factor alpha receptor (PDGFαR) exhibited nuclear Myt1 immunoreactivity (85.22% in PBS controls; 77.46% in MHV). The density of cells double immunolabeled for Myt1 and PDGFαR was significantly increased in sections from MHV mice as compared to PBS injected control mice.

As expected during early remyelination, the density of oligodendrocytes is significantly lower in MHV mice as compared with PBS controls, and among the mature oligodendrocytes immunolabeled with CC1 only a small proportion exhibited Myt1 nuclear immunoreactivity (Figure 5; PBS = 3.15%; MHV = 5.35%). The small proportion of Myt1+ CC1+ double labeled cells may reflect the lineage relationship of

cells in transition from the OP stage into mature oligodendrocytes since during CNS development Myt1 immunoreactivity is downregulated after OP cells undergo terminal differentiation into mature oligodendrocytes (Armstrong et al., 1995).

A substantial population of cells expressing Myt1 did not appear to be OP cells (*i.e.* Myt1+ PDGFαR-) yet was not equivalent to the much lower density of cells that had acquired CC1 immunolabeling as oligodendrocytes (*i.e.* Myt1+ CC1+). Therefore, immunolabeling for Musashi1 (Msi1) was used as a marker of adult neural stem cells prior to the OP cell stage (Sakakibara and Okano, 1997; Pincus et al., 1998; Kaneko et al., 2000). A population of cells with nuclear immunoreactivity for Myt1 was also immunolabeled for Msi1, and the cell density of these Myt1+ Msi1+ cells was increased in lesioned white matter (Figure 6A, D). Msi1 is known to be expressed in reactive astrocytes (Sakakibara and Okano, 1997), which was confirmed in the MHV lesioned tissue using double immunolabeling for glial fibrillary acidic protein (GFAP), which is a marker of reactive astrocytes (Figure 6B). However, reactive astrocytes immunolabeled for GFAP did not exhibit Myt1 immunoreactivity (Figure 6C). Therefore, the reactive astrocytes did not complicate the interpretation of the Myt1 immunolabeled cells as neural stem cells based upon Msi1 identification.

The overall population of Myt1 expressing cells can be appreciated by combining the cell densities quantified for each cell type from MHV lesions at 4 wpi as compared to PBS control white matter (Figure 7). Among these populations, Myt1 expression appears to begin during the neural stem cell stage (Myt1+ Msi1+) and continue throughout the OP cell stage (Myt1+ PDGFαR+), which comprises the majority of cells with nuclear Myt1 immunoreactivity. In contrast, Myt1 is expressed in only a small proportion of the

mature oligodendrocyte population (Myt1+ CC1+), possibly indicating expression during a transitional stage of OP differentiation into mature oligodendrocyte with subsequent down regulation.

MHV lesions also contain substantial populations of infiltrating and endogenous immune cells which were also evaluated and found to be negative for Myt1 immunoreactivity (Figure 8). Myt1 was not detected in Mac1 immunolabeled activated macrophage/microglial cells (Figure 8A), or within lymphocytes immunostained with either Ox-52 for T-cells (Figure 8B) or CD-45 receptor for B-cells (data not shown).

Myt1 expression in cuprizone induced demyelination of the corpus callosum.

The increased expression of Myt1 in response to MHV induced demyelination was also observed with cuprizone induced demyelination of the corpus callosum (Figure 9). These two experimental models have distinctly different lesion characteristics yet both undergo spontaneous remyelination after a transient episode of demyelination (see discussion). A period of 5 weeks of cuprizone ingestion results in extensive demyelination of the corpus callosum and corresponds with the peak of the OP proliferation response (Armstrong et al., 2002). This 5 week time point in the cuprizone model was examined to compare with the observed increase of Myt1 associated with proliferation in the MHV model. In the cuprizone induced lesions, the density of cells with detectable Myt1 mRNA transcripts was markedly increased and corresponded with a high level of BrdU incorporation, indicative of cell proliferation (Figure 9).

Myt1 expression in MS lesions and control cases.

In the normal human CNS, Myt1 expression is highest during development and down-regulated in adults (Kim et al., 1992). However, Myt1 expression is maintained in cells of the adult human subventricular zone, indicating persistent expression in immature cell populations (Armstrong et al., 1997). These findings together with our analysis of Myt1 expression in experimental remyelination led us to examine Myt1 expression in multiple sclerosis (MS) lesions as an indication of a potential role of Myt1 in human demyelinating diseases.

MS lesions of differing stages of demyelinating activity (active, inactive, remyelinated) and control cases were analyzed by immunostaining and *in situ* hybridization to detect Myt1 expression (Figure 10 and Table I). In MS cases, Myt1 nuclear immunoreactivity was frequently observed in cells located in the PPWM adjacent to active or inactive lesions, or within early remyelinating lesions (Figure 10A and Table I). In control adult white matter, nuclear Myt1 immunostaining was much less frequently observed (Figure 10B and Table I), consistent with our previous report (Armstrong et al., 1997). In a minority of lesions, nuclear Myt1 immunoreactivity was detected among infiltrating lymphocytes (data not shown) or reactive astrocytes had cytoplasmic Myt1 immunoreactivity (Figure 10C). No immunostaining was detected above background levels when the Myt1 primary antibody was omitted or was replaced with an antibody against Myt1L, a closely related Myt1 family member that is not expressed in oligodendrocytes (Kim et al., 1998 and data not shown). The increased frequency of Myt1 immunostaining in MS tissues was confirmed using *in situ* hybridization to detect Myt1 mRNA transcripts (Figure 10D).

Myt1 expression may activate transcription of myelin-specific genes as OP cells differentiate into mature oligodendrocytes (Kim et al., 1992; Nielsen et al., 2004). To identify cells with nuclear Myt1 localization relative to myelin-specific gene transcription, immunostaining for Myt1 was combined with *in situ* hybridization for proteolipid protein mRNA (Figure 10E, F). In the combined protocol, specific signal was maintained although the number of cells identified was reduced, relative to the separate detection methods. Individual cells could be clearly identified as double labeled for Myt1 immunoreactivity and PLP mRNA transcripts in MS tissues (Figure 10E and inset), which was not observed in the white matter of control cases (Figure 10F). A similar protocol to double label OP cells for PDGFαR mRNA and Myt1 was not feasible because of the low abundance of PDGFαR transcripts per cell. Importantly, as in the MHV data of Myt1+ CC1+ cells (Figures 5, 7), Myt1 expression in a subset of oligodendrocytes identified by PLP mRNA may indicate ongoing OP differentiation into mature oligodendrocytes and support a potential role for Myt1 in oligodendrocyte regeneration in MS lesions.

DISCUSSION

In experimental and human demyelinating diseases, the CNS is capable of effective oligodendrocyte regeneration, remyelination, and recovery of function in viable axons. However, this capacity for repair becomes limited with repeated or chronic episodes of demyelination. Therefore, to better understand how successful remyelination can be accomplished, the current study examines the cellular responses during spontaneous remyelination following transient experimental demyelination. Recent

studies have identified an emerging array of transcription factors that are associated with oligodendrocyte progenitor proliferation, induction of differentiation toward an oligodendrocyte phenotype, and elevated transcription of myelin-specific genes during myelination (Gohkan et al., 2005; Wegner and Stolt, 2005; Ligon et al., 2006). Analysis of developmental myelination has predicted specific transcription factors that have been shown to be involved in oligodendrocyte lineage responses to demyelination (Arnett et al., 2004; Fancy et al., 2004; Watanabe et al., 2004).

Myt1 warranted analysis as a transcription factor regulating oligodendrocyte regeneration and remyelination based upon findings indicating a role in proliferation, differentiation, and activation of myelin-specific gene transcription during the generation of oligodendrocytes in the developing CNS (Armstrong et al., 1995; Nielsen et al., 2004). A role for Myt1 in human development has been predicted from the pattern of Myt1 expression in the developing human brain and from correlation of Myt1 immunolabeling with the attempted regenerative response of immature oligodendrocyte lineage cells in cases of periventricular leukomalacia (Hirayama et al., 2003). In the adult human CNS, Myt1 is expressed in germinal zones and is upregulated in gliomas, indicating potential function in immature proliferative cell types (Armstrong et al., 1997).

Using two distinctly different mouse models of demyelination, we report increased expression of Myt1 as a local white matter response to demyelination. Further analysis used the MHV model of spinal cord demyelination to assess the oligodendrocyte regenerative response in the context of a complex lesion environment that includes gliosis, inflammation, and breakdown of the blood-brain barrier to reflect the complex pathology of MS lesions (Lucchinetti et al., 2005; Morales et al., 2006). Myt1 expression

corresponded with incorporation of BrdU, indicative of a proliferative phenotype. Consistent with this finding, the majority of cells with nuclei immunolabeled for Myt1 were double immunolabeled for PDGFαR, a marker of OP cells, with a marked response also noted in cells immunolabeled for Msi1, a marker of neural stem cells. Furthermore, a role for Myt1 in the oligodendrocyte lineage response in human injury and disease is suggested by our observation of increased expression of Myt1 associated with MS lesions.

In the current study, a role for Myt1 in remyelination is supported by correlation with increased expression of Myt1 in proliferating cells and localization of Myt1 in nuclei of neural stem cells and OP cells. Interestingly, only nuclear immunoreactivity for Myt1 was observed in the adult mouse tissues, even though a transition to the cytoplasm was observed in developing oligodendrocytes (Armstrong et al., 1995). The present quantitative cellular analysis demonstrating Myt1 expression in OP cells extends an earlier report of increased abundance of mRNA transcripts for both Myt1 and PDGFαR during remyelination following ethidium bromide induced demyelination in rats (Sim et al., 2002). In addition, similar to our current quantification of Myt1 and BrdU incorporation, our previous MHV study demonstrated that PDGFαR+ cell proliferation in response to demyelination was localized within and near lesions (Redwine and Armstrong, 1998). However, Myt1 is expressed in additional populations of responding cells that were not identified by PDGFαR immunolabeling. We identified a subset of Myt1 expressing cells by co-immunolabeling for Msi1. Msi1 is an RNA-binding protein that has been used as a marker of neural stem cells in the developing and adult CNS of rodents and humans (Sakakibara and Okano, 1997; Pincus et al., 1998; Kaneko et al.,

2000). Although Msi1 is expressed in reactive astrocytes (Sakakibara and Okano, 1997), in MHV lesions Myt1 was not expressed in astrocytes so that Msi1 cells that express Myt1 are likely to be neural stem cells. Therefore, the initial expression of Myt1 in MHV lesions is expected to be in neural stem cells that may represent a relatively small population of cells that is responsive to demyelination in addition to the OP population characterized by PDGFαR expression.

While the current analysis has not directly demonstrated a lineage progression for the expression of Myt1 during MHV disease progression, a possible scenario from current data and the available literature is that Myt1 is expressed in Msi1+ neural stem cells that may differentiate into PDGFαR+ OP cells then amplify in response to demyelination to generate CC1+ oligodendrocytes to accomplish remyelination. Among these lineage stages, the endogenous OP population is highly proliferative and may be replenished by neural stem cells. This scenario is based on association of the proliferative response with the OP stage in MHV lesions, other models of experimental demyelination, and MS lesions (Redwine and Armstrong, 1998; Reynolds et al., 2001; Solanky et al., 2001; Watanabe et al., 2002). Myt1 expression prior to the OP stage has been shown with *in vitro* analysis of cultured neonatal cells (Armstrong et al., 1995). *In vitro*, Myt1 continued to be expressed at the OP stage followed by down-regulation in mature oligodendrocytes after accumulation of myelin proteins (Armstrong et al., 1995). In the current MHV study, expression of Myt1 was observed in a small proportion of CC1+ oligodendrocytes, which may be cells that recently differentiated and had not yet down-regulated Myt1. In a study of spinal cord injury in adult rats, CC1+ cells were reported as not expressing Myt1 (Wrathall and Hudson, 1998). This difference between reports of

CC1 and Myt1 double-labeling may reflect the time points at which the tissue was analyzed, methodological differences of immunodetection, or the relatively small population of double-labeled cells that may be missed in a qualitative analysis.

The current finding that the increased density of Myt1 cells is specifically localized to areas of white matter lesions is consistent with the local generation of oligodendrocytes reported in several studies of remyelination. In focally demyelinated spinal cord, newly generated remyelinating cells appeared to be generated from a local ring of normal tissue surrounding the lesion (Franklin et al., 1997). Similarly, retroviral labeling of remyelinating cells indicated recruitment from less than 500 microns from the lesion site in adult rat corpus callosum (Gensert and Goldman, 1997). The signals in the lesion environment that can induce proliferation of immature endogenous cells to regenerate oligodendrocytes are of great interest to identify. PDGF has been shown to be synthesized by reactive astrocytes in MHV lesions (Redwine and Armstrong, 1998) and so could be a mitogen for OP cells prior to oligodendrocyte repopulation of lesions. Indeed, in cuprizone demyelinated mice, haploinsufficiency of PDGFαR resulted in reduced proliferation of OP cells in lesioned corpus callosum and impaired oligodendrocyte regeneration (Murtie et al., 2005). Additional signals are likely to be present in lesions and also influence neural stem cell responses in white matter along with the OP response.

Increased expression of Myt1 in lesions was observed in distinctly different experimental models, possibly indicating regulation by signals that are common to an environment of oligodendrocyte loss and demyelination. MHV lesions involve oligodendrocyte cell loss and a robust immunological response, including infiltration of

CD8+ and CD4+ T cells, B lymphocytes producing immunoglobulins, macrophages, and reactive glial cells (Redwine and Armstrong, 1998; Matthews et al., 2002). MHV involves a complex inflammatory response as observed in types I and II of MS pathology (Lucchinetti et al., 2000). In contrast, the cuprizone model involves oligodendrocyte apoptosis and microglial activation in the absence of marked lymphocytic infiltration (Matsushima and Morell, 2001). These are features observed in type III and IV MS lesions and have also been observed in lesions from cases of rapidly deteriorating MS (Lucchinetti et al., 2000; Barnett and Prineas, 2004). Comparison of the results in the cuprizone and MHV models, which differ in the extent of lymphocytic infiltration, suggests that lymphocytes are unlikely to be responsible for the localized increase of cells expressing Myt1 and points to other components of the lesion environment.

Analysis of MS lesions demonstrated the highest density of Myt1 expressing cells in early remyelinating lesions, consistent with our findings in MHV and cuprizone. Furthermore, Myt1 expression was increased in PPWM relative to active or inactive lesion areas. The variability of Myt1 expression in MS lesions may reflect the reported differences of immature OP populations (Maeda et al., 2001; Chang et al., 2002). In contrast to MHV lesions, some MS lesions exhibited Myt1 immunoreactivity in lymphocytes and astrocytes. In the unusual cases in which astrocytes expressed Myt1, the immunoreactivity was localized in the cytoplasm. Similarly, Myt1 immunoreactivity in astrocyte cytoplasm was observed in highly reactive astrocytes of epileptic foci in human temporal lobe tissue (Armstrong et al., 1997). It is not clear whether this astrocytic expression of Myt1 is the result of a species difference or possibly due to differences in lesion pathology.

Further work is required to understand the regulation of Myt1 in development and pathology, and the function of Myt1 in each context. In addition, Myt1 may act in concert with other transcription factors or co-factors that contribute to transcriptional control of oligodendrocyte lineage responses to demyelination. Myt1 has been shown to form complexes with Sin3B, a protein that mediates repression by recruiting histone deacetylases (Romm et al., 2005). However, Myt1 acted as a transcriptional activator of a myelin gene in transient transfection assays (Nielsen et al., 2004). Therefore, the function of Myt1 may depend upon the levels of other nuclear proteins, such as Sin3B, that may be differentially regulated in cells relative to stage of differentiation or environmental context. The current study documenting Myt1 expression corresponding to regenerative responses of the oligodendrocyte lineage in animal models and MS lesions suggests that Myt1 may have functional importance in remyelination.

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Figure 1

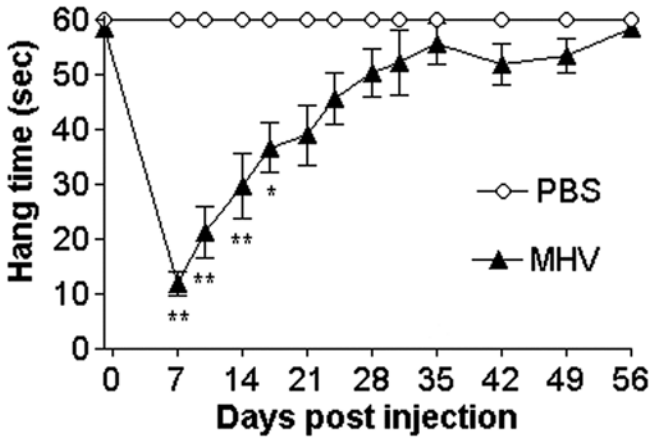

Figure 1. ***Disease progression among MHV infected mice used in the study.*** Motor impairment and recovery was monitored with the hang time test of grip strength and coordination. Control mice injected with PBS can readily hang from bars of a wire cage top for 60 seconds. Following MHV injection, severely affected mice had significantly reduced hang times when tested on days 7, 10, 14, and 17. In the following weeks, the hang time scores improve gradually until reaching control levels. ANOVA with repeated measures: **$p < 0.01$; *$p < 0.05$; Days 7, 10, 14: n = 23 mice for MHV, n = 12 mice for PBS; Days 17, 21, 24, 28: n = 17 mice for MHV, n = 9 mice for PBS; Days 35, 42, 49, 56: n = 9 mice for MHV, n = 6 mice for PBS.

Figure 2

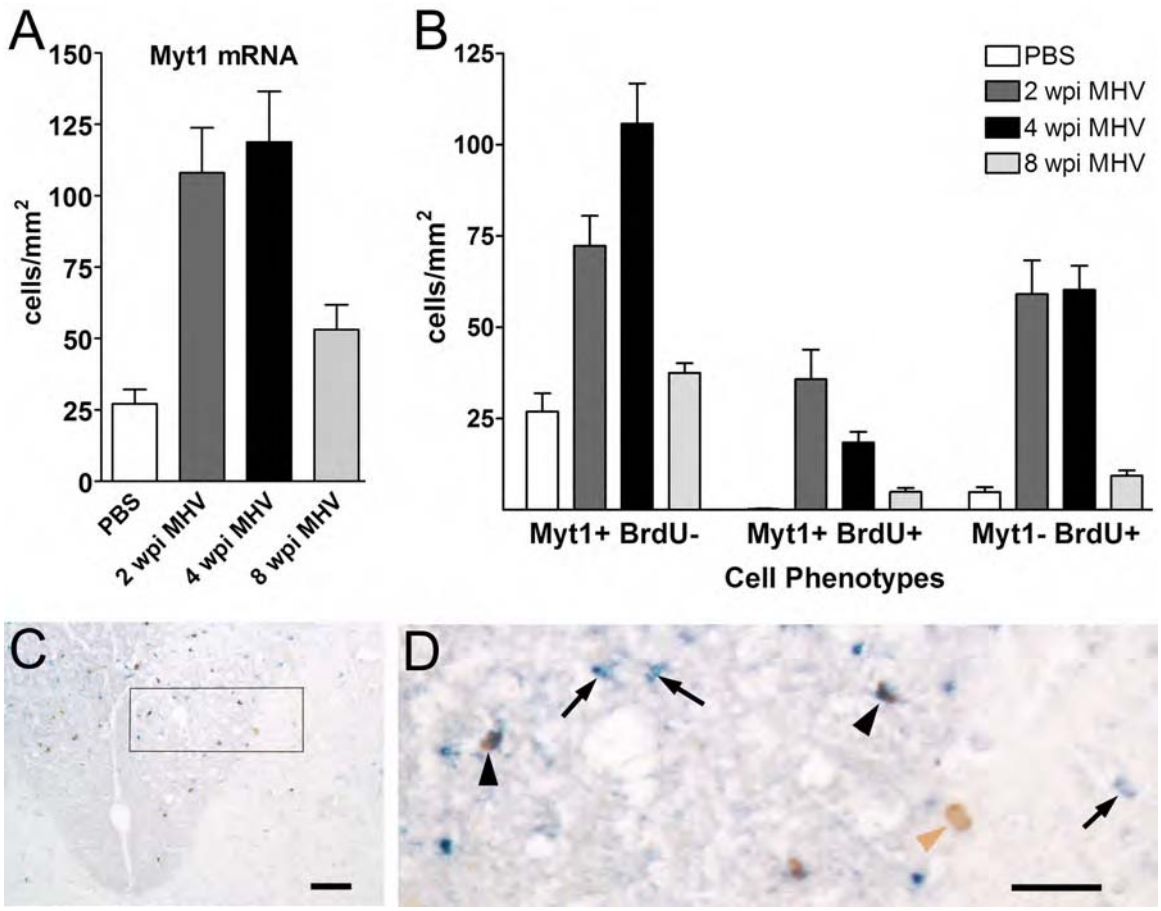

Figure 2. ***Quantification of Myt1 mRNA expression and BrdU incorporation at distinct stages of MHV disease progression.*** Mice were injected intracranially with MHV or PBS vehicle and perfused after 2 weeks post injection (wpi) to correspond with the active demyelination stage, 4 wpi to correspond with early remyelination, and 8 wpi to correspond with more advanced remyelination. **A**: In MHV injected mice, as compared to PBS control mice, the density of cells labeled by *in situ* hybridization for Myt1 mRNA is increased by 2wpi ($p = 0.0323$), peaks at 4 wpi ($p = 0.0020$), and then declines by 8 wpi but is still elevated from controls ($p = 0.0297$). **B**: BrdU was administered during the final 4 hours prior to sacrifice to identify actively proliferating cells (BrdU+) among the population of cells expressing Myt1 mRNA (Myt1+). The Myt1+ BrdU+ cell density peaks during active demyelination (2 wpi; $p = 0.016$) then remains significantly increased at 4 wpi ($p = 0.003$), relative to the very low density (0.4 cells per mm^2) in PBS injected mice. By 8 wks, Myt1+ BrdU+ cell density has returned

to control levels (p = 0.0791). BrdU+ cells that do not have detectable Myt1 mRNA (Myt1-) are increased over control levels at 2 wpi (p = 0.016) and at 4 wpi (p = 0.0007), but are still slightly elevated at 8 wks (p = 0.017). PBS, n = 3 mice; MHV 2 wpi, n = 4 mice; MHV 4 wpi, n = 6 mice; MHV 8 wpi, n = 4 mice. **C**: Representative image of dorsal column area in transverse section of cervical spinal cord from an MHV infected mouse at 4 wpi. *In situ* hybridization showing Myt1 mRNA transcripts (blue) combined with detection of BrdU (brown) to assess proliferation. The lesioned areas of the dorsal columns (upper part of image) show an increase of BrdU incorporation and an increased density of cells expressing Myt1, compared to the normal appearing areas in the deep dorsal column white matter. **D**: Enlargement of area boxed in panel C. Cells with detectable Myt1 mRNA are small with an elongated cell body, characteristic of oligodendrocyte progenitor cells, and could be found labeled with BrdU (Myt1+ BrdU+, black arrowheads) or may not have incorporated BrdU during this short 4-hour terminal pulse (Myt1+ BrdU-, black arrows). BrdU labeled cells that do not express Myt1 (Myt1- BrdU+, brown arrowhead) are also common, such as the example with a large oval nucleus that is characteristic of an astrocyte. Scale bars, C = 50 μm, D = 25 μm.

Figure 3

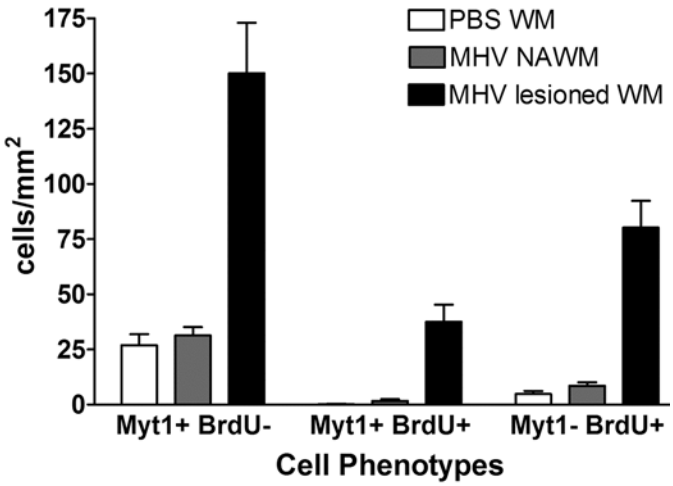

Figure 3. ***Quantification of Myt1 mRNA expression and BrdU incorporation within normal versus lesioned white matter.*** Within transverse spinal cord sections, regions were demarcated as white matter areas of demyelination (MHV lesioned WM), adjacent normal appearing white matter (MHV NAWM), or white matter of PBS-injected controls (PBS WM). An increased density of cells with detectable levels of Myt1 mRNA transcripts is mainly restricted to MHV lesion areas, which are significantly elevated compared with NAWM ($p = 0.0005$) or with PBS injected control white matter ($p = 0.0081$). Myt1+ BrdU+ proliferating cells are also significantly increased specifically within lesion areas relative to NAWM ($p = 0.0009$) and PBS control white matter ($p = 0.0003$). Myt1- BrdU+ cells within MHV lesions are also significantly increased over both NAWM ($p = 0.0002$) and PBS control white matter ($p = 0.0038$). NAWM was not different from PBS WM for the densities of Myt1+ BrdU- cells ($p = 0.518$), Myt1+ BrdU+ cells ($p = 0.2734$) or Myt1- BrdU+ cells ($p = 0.1821$). PBS, n = 3 mice; MHV, n = 6 mice.

Figure 4

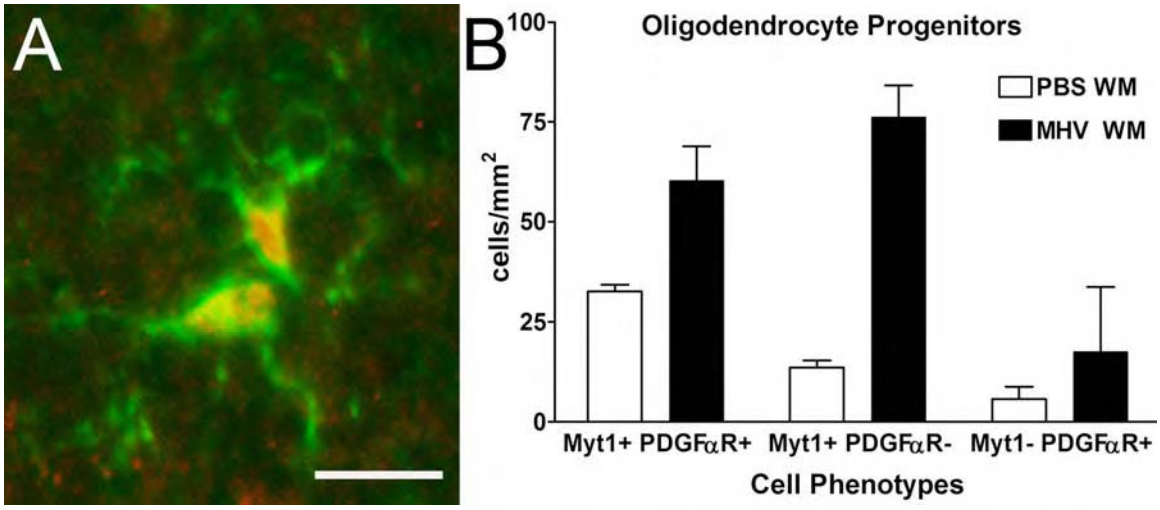

Figure 4. ***Quantification of nuclear Myt1 immunoreactivity in oligodendrocyte progenitors.*** **A**: Representative image of two cells that are double immunolabeled for Myt1 (red nuclei) and the oligodendrocyte progenitor (OP) marker PDGFαR (green). Scale bar = 10 μm. **B**: Quantification of immunostained cells in the total spinal cord white matter (WM) in transverse sections of mice perfused 4 weeks after injection of PBS or MHV, which corresponds to an early remyelination phase of the disease progression. The density of cells with nuclear immunostaining for Myt1 is greatly increased in MHV WM compared to PBS injected control WM (Myt1+ PDGFαR+, $p = 0.0207$; Myt1+ PDGFαR-, $p = 0.0018$; PBS, $n = 3$ mice; MHV, $n = 3$ mice). Among cells identified as OPs by PDGFαR immunostaining (PDGFαR+), the large majority had nuclear Myt1 immunoreactivity (Myt1+ PDGFαR+) and relatively few did not have detectable Myt1 (Myt1- PDGFαR+).

Figure 5

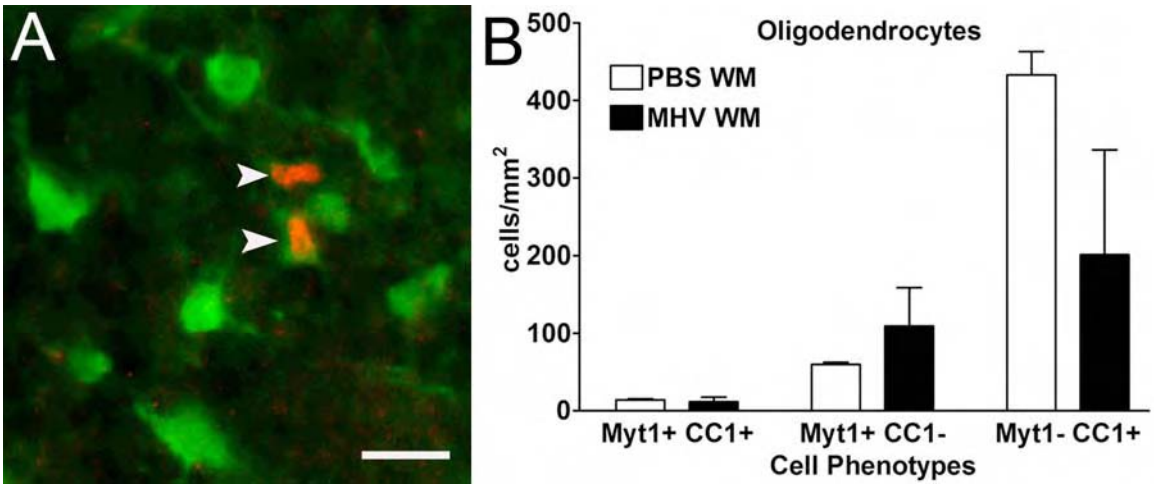

Figure 5. ***Quantification of nuclear Myt1 immunoreactivity in oligodendrocytes.***
A: Representative image showing mature oligodendrocytes, identified with CC1 immunolabeling (green) in spinal cord white matter (WM) from a PBS injected control mouse. Nuclear immunoreactivity for Myt1 (red) is evident in two cells (arrowheads), one of which has cytoplasmic CC1 immunolabeling (lower arrowhead). Scale bar = 10 μm. **B**: Quantification of immunostained cells in spinal cord WM in transverse sections of mice perfused at 4 weeks after injection of PBS or MHV. Among cells identified as differentiated oligodendrocytes by CC1 immunostaining, a large majority did not express detectable Myt1 immunoreactivity (Myt1- CC1+). As expected in white matter undergoing remyelination, the density of mature oligodendrocytes (Myt1- CC1+) is reduced in MHV injected mice compared to PBS control WM ($p = 0.013$). PBS, n = 3 mice; MHV, n = 3 mice.

Figure 6

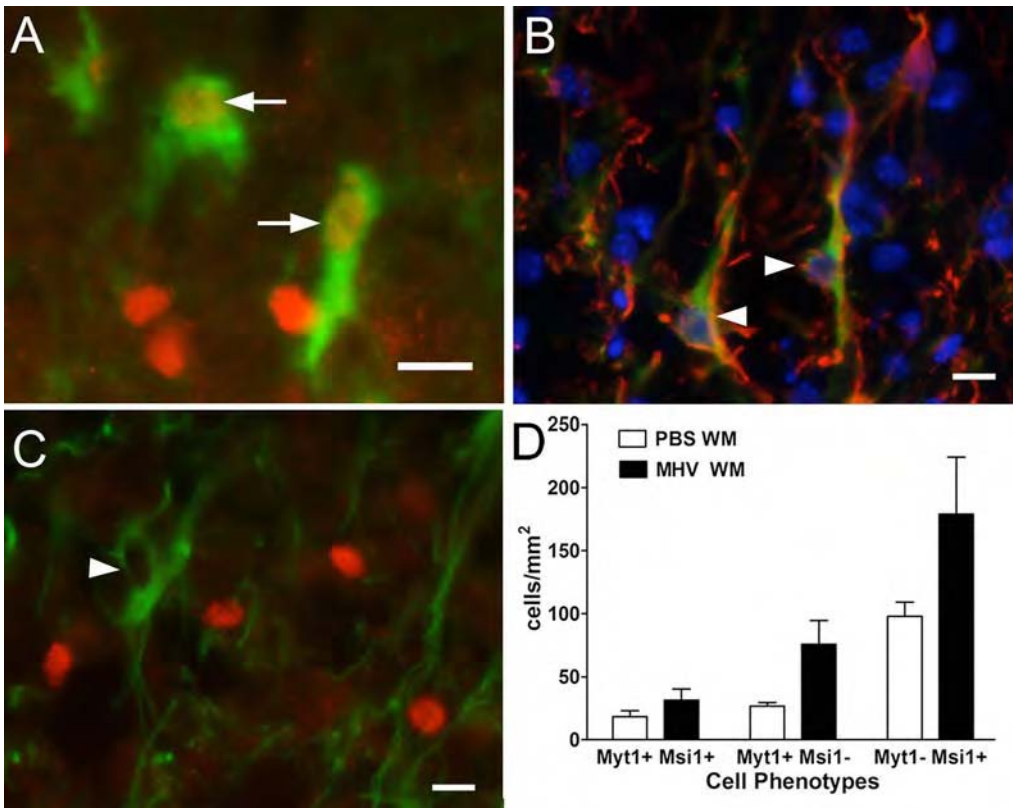

Figure 6: ***Quantification of nuclear Myt1 immunoreactivity in neural stem cells.*** **A, B, C:** Immunostaining of white matter lesions in sections from spinal cords of MHV mice at 4 wpi, corresponding with early remyelination. **A**: Representative images showing cells immunolabeled with the neural stem cell marker Musashi1 (Msi1; arrows, green) that also exhibit nuclear immunoreactivity for Myt1 (red). In addition, three cells exhibit nuclear Myt1 immunoreactivity but not Msi1 immunolabeling. **B**: Msi1 immunoreactivity (green) is present in subpopulations of astrocytes (arrowheads) identified by immunolabeling for glial fibrillary acidic protein (GFAP; red). Nuclei are labeled with DAPI (blue). **C:** Astrocytes immunolabeled for GFAP (green, arrowhead indicates the cell body) do not exhibit nuclear Myt1 immunoreactivity (red). This finding allows the interpretation of cells in panel A (arrows) as neural stem cells, based upon immunolabeling for Musashi1 (expressed in neural stem cells and astrocytes) in combination with nuclear Myt1 immunoreactivity (not found in astrocytes). In addition, four cells exhibit nuclear Myt1 immunoreactivity but not Msi1 immunolabeling. **D**: Quantification of Myt1 and Msi1 immunolabeling in white matter (WM) of spinal cord

sections from mice sacrificed at 4 weeks after injection of MHV or PBS vehicle. A relatively small population of cells, interpreted as potential neural stem cells, was double labeled for nuclear Myt1 immunoreactivity and Msi1 immunolabeling (Myt1+ Msi1+) in PBS WM and was increased but not significantly different in MHV WM. PBS, n = 3 mice; MHV, n = 3 mice. Scale bars in A, B, C = 10 μm.

Figure 7

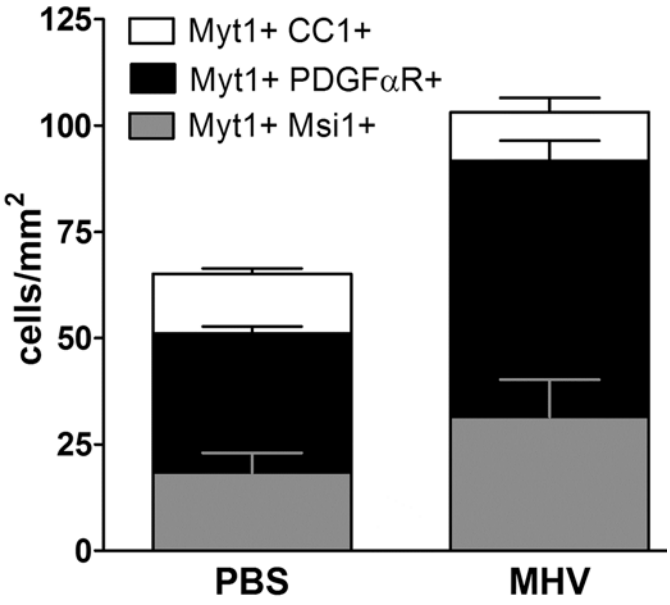

Figure 7. ***Combined data for comparison among cell types exhibiting nuclear Myt1 immunoreactivity in PBS control mice and MHV mice at 4 weeks post infection*****.** OPs immunolabeled with PDGFαR were the principal cell type that expressed Myt1 (MHV = 60.2 $\pm$ 4.7 cells/mm^2; PBS = 32.6 $\pm$ 1.7 cells/mm^2). Musashi1 (Msi1) neural stem cell marker detected a smaller population of Myt1 expressing cells (MHV = 31.4 $\pm$ 8.7 cells/mm^2; PBS = 18.3 $\pm$ 4.6 cells/mm^2). Relatively few cells exhibited nuclear Myt1 immunoreactivity after differentiating into mature oligodendrocytes, as indicated by CC1 immunolabeling (MHV = 11.4 $\pm$ 3.4 cells/mm^2; PBS = 14.1. $\pm$ 1.3 cells/mm^2). Data generated from quantification shown in Figures 5, 6, 7 to facilitate analysis of Myt1 expression across phenotypes.

Figure 8

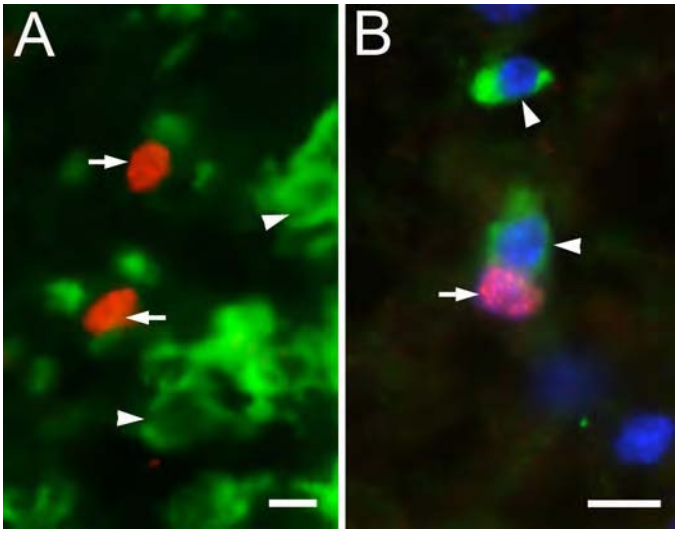

Figure 8. ***Myt1 is not expressed in immune cells of control or MHV-lesioned spinal cord.*** **A, B**: Representative images from sections of spinal cord white matter from mice sacrificed 4 weeks post infection with MHV. **A**: Macrophages/microglia immunolabeled for Mac1 (green, arrowheads) do not show nuclear immunoreactivity for Myt1 (red, arrows). **B**: T-lymphocytes immunolabeled for Ox52 (green, arrowheads), a pan T-cell marker, do not show nuclear immunoreactivity for Myt1 (red, which appears pink due to overlap with the blue DAPI nuclear stain, arrow). Scale bars in A, B = 10 μm.

Figure 9

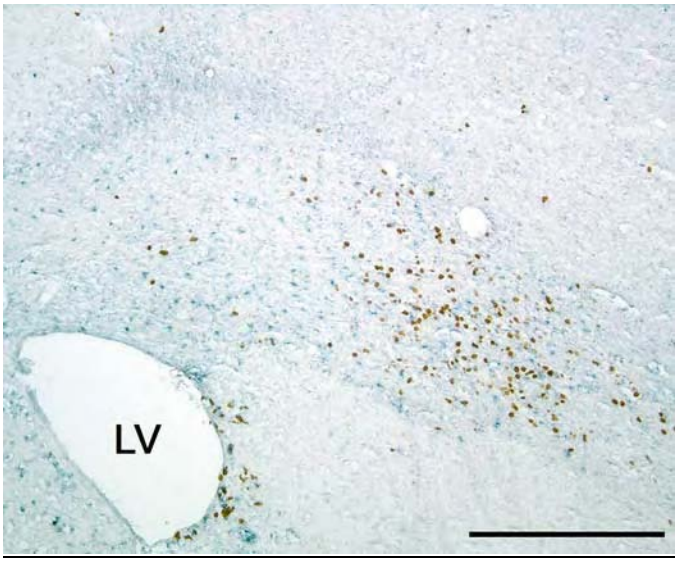

Figure 9. ***Myt1 mRNA expression and BrdU incorporation in cuprizone demyelinated corpus callosum.*** *In situ* hybridization to detect Myt1 mRNA (blue) combined with immunohistochemistry for BrdU (brown) in coronal sections of C57Bl/6 mice. After 5 weeks of cuprizone ingestion, lesioned areas of the corpus callosum (CC) typically exhibit a high density of cells incorporating BrdU. This lesion area also has a high density of cells expressing Myt1 mRNA transcripts. LV = lateral ventricle. Scale bar = 300 μm.

Figure 10

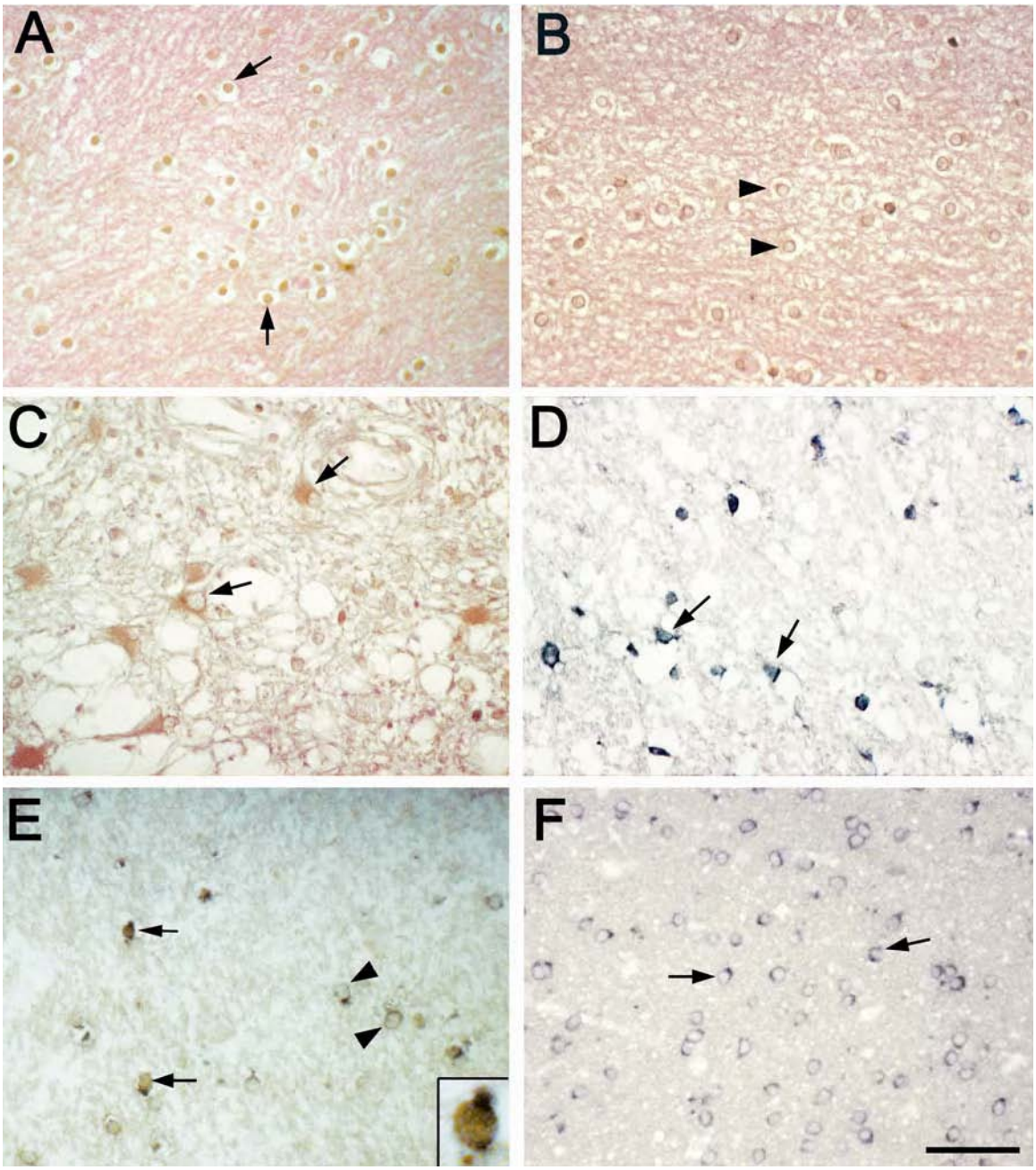

Figure 10. ***Myt1 expression in multiple sclerosis (MS) and control cases.***
Representative results are shown from adult human brain sections that were immunostained for Myt1 (**A,B,C**), processed for *in situ* hybridization for Myt1 mRNA (**D**), or analyzed by immunostaining for Myt1 combined with *in situ* hybridization for proteolipid protein (PLP) mRNA as a marker of oligodendrocytes (**E,F**). Nuclear immunostaining for Myt1 (brown DAB with pink eosin tissue counter stain) was seen in

small round cells in the periplaque white matter (PPWM) adjacent to MS lesions (A, PPWM; case 9, Table 1), at an increased density relative to white matter control areas (B; case 2, Table 1). In some MS lesions (C, lesion area; case 6, Table 1), reactive astrocytes exhibited cytoplasmic immunoreactivity for Myt1 (brown DAB with pink eosin tissue counter stain), which was never seen in control cases. *In situ* hybridization for Myt1 mRNA (D, lesion edge; dark blue; case 4, Table 1) in MS lesions indicated expression of Myt1 in a pattern similar to Myt1 immunoreactivity (A). Within MS lesions, some cells with nuclear Myt1 expressed PLP mRNA transcripts (E, lesion; Myt1 brown, PLP dark blue; case 10, Table 1). Co-labeling for Myt1 among oligodendrocytes expressing PLP mRNA was generally not observed in adult human control white matter (F; case 3, Table 1). Scale bars = 50 μm for A-F, as shown in F. A-F: Arrows indicate examples of cells expressing Myt1 protein (A, B, C, E) or mRNA transcripts (D). Arrowheads (B, E, F) indicate examples of cells that are not expressing Myt1.

Table I.

Density of cells with Myt1 nuclear immunoreactivty in white matter of human controls and MS lesions.

Case	Lesion Stage	Lesion cells/mm2	PPWM cells/mm2	NWM cells/mm2
1.	control			15
2.	control			101
3.	control			86
4.	Demyelinated inactive	101	321	
5.	Demyelinated inactive	113	284	
6.	Demyelinated inactive	57	309	
7.	Early active	193	371	
8.	Early active	113	365	
9.	Early active	38	309	
10.	Early remyelination	649	n/a	
11.	Early remyelination	536	n/a	

PPWM = periplaque white matter adjacent to lesions in MS tissues.
NWM = normal white matter in non-neurological controls.
n/a = Samples are each from a biopsy of the lesion area so PPWM tissue is not available.

CHAPTER 3

Platelet-derived Growth Factor (PDGF) Promotes Repair of Chronically Demyelinated White Matter

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ABSTRACT

In multiple sclerosis, remyelination becomes limited following repeated or prolonged episodes of demyelination. Factors influencing this failure to remyelinate are important to identify because denuded axons have impaired neurotransmission and increased vulnerability to transection. Studies using the cuprizone model of chronic demyelination in mice have shown that limited remyelination is associated with depletion of oligodendrocyte progenitor (OP) cells and inhibition of OP differentiation in lesions. We now show that apoptosis continues after removal of cuprizone from the diet and is an additional factor contributing to the limited remyelination observed following chronic demyelination. To test the effect of platelet-derived growth factor-A (PDGF-A) in recovery from chronic cuprizone demyelination we used *hPDGF-A* transgenic (tg) mice with human *PDGF-A* under control of an astrocyte-specific promoter. Following chronic demyelination and removal of cuprizone from the diet, remyelination improved significantly in *hPDGF-A* tg mice compared with wild-type mice. PDGF-A was expected to act as an OP mitogen to counter OP depletion during chronic demyelination and promote remyelination. However, *hPDGF-A* tg mice OP density and proliferation values were increased during acute demyelination but not during chronic demyelination or the subsequent recovery period. Importantly, *hPDGF-A* tg mice had improved oligodendroglial regeneration associated with reduced apoptosis in the corpus callosum during the recovery period. Therefore, a significant effect of PDGF-A *in vivo* may be as a survival factor during the generation of remyelinating oligodendrocytes. Furthermore, preventing apoptosis may be important not only during active demyelination but also for supporting oligodendrocyte regeneration and remyelination of chronic lesions.

INTRODUCTION

CNS demyelination results from multiple sclerosis (MS), toxic insults, leukoencephalopathies, vascular lesions, and traumatic injury. Loss of myelin impairs action potential conduction and increases the vulnerability of axons to atrophy and transection. In MS, the most prevalent demyelinating disease, remyelination becomes limited with repeated or chronic episodes of demyelination (Ozawa et al., 1994). Factors leading to the eventual inability to remyelinate chronic MS lesions are not well understood.

The pathology of MS lesions is heterogeneous and the effect of MS on the oligodendrocyte lineage population varies dramatically (Lucchinetti et al., 1999, 2000). Oligodendrocyte progenitor (OP) cells and premyelinating oligodendrocytes can persist in MS lesions yet fail to efficiently remyelinate denuded axons (Wolswijk 2000, 2002; Maeda et al., 2001; Chang et al., 2002). With prolonged disease duration, depletion of the OP and premyelinating oligodendrocyte populations may limit the capacity for remyelination of chronic lesions (Reynolds et al., 2002; Wolswijk 2000, 2002; Mason et al., 2004). OP cells in MS lesions and experimental demyelination have been identified by expression of NG2 proteoglycan and platelet-derived growth factor alpha receptor (PDGFαR) (Redwine and Armstrong, 1998; Solanky et al., 2001; Chang et al., 2002; Wilson et al., 2006). PDGF-A ligand activation of PDGFαR signaling can stimulate OP proliferation in response to acute experimental demyelination (Frost et al., 2003; Woodruff et al., 2004; Murtie et al., 2005) but this signaling pathway has not yet been tested in the context of chronic demyelination.

The current study examines the effect of *PDGF-A* transgene expression on the potential of endogenous OP cells to generate remyelinating oligodendrocytes in chronically demyelinated lesions. Cuprizone ingestion in mice is used to induce chronic demyelination of the corpus callosum with limited remyelination, even after removal of cuprizone from the diet (Tansey et al., 1996; Armstrong et al., 2006). Following cuprizone induced chronic demyelination, axons remain viable and can be remyelinated by transplanted OP cells (Mason et al., 2004). We analyzed this chronic lesion model using *hPDGF-A* transgenic (tg) mice with the human *PDGF-A* gene under control of the astrocytic glial fibrillary acidic protein (GFAP) promoter (Fruttiger et al., 2000). Since reactive astrocytes are the main source of endogenous PDGF-A in demyelinated lesions (Redwine and Armstrong, 1998), regulated overexpression of PDGF-A was exhibited in reactive astrocytes of *hPDGF-A* tg mice. Mice were examined throughout acute and chronic cuprizone demyelination followed by a recovery period on a normal diet. Surprisingly, we show that apoptosis continues in lesion areas after the recovery period on normal chow. This cell death may contribute to the limited capacity of OP cells to generate remyelinating oligodendrocytes in a chronic lesion environment. In *hPDGF-A* tg mice, apoptosis was reduced during the recovery period and the extent of remyelination was increased relative to wild-type mice. Therefore, a significant effect of PDGF-A *in vivo* may be as a survival factor in the context of regeneration of oligodendrocytes in chronic lesions. Furthermore, preventing apoptosis may be important not only during active demyelination but also for remyelination of chronic lesions.

MATERIALS AND METHODS

Animals

Mice were bred and maintained in the Uniformed Services University of the Health Sciences (USUHS) animal housing facility in accordance with guidelines of the National Institutes of Health and the USUHS Institutional Animal Care and Use Committee. Hemizygous *hPDGF-A* tg mice on a C57Bl6/cba hybrid background were generously provided by Dr. Marcus Fruttiger (University College London; Fruttiger et al., 2000). Hemizygous *hPDGF-A* tg mice were bred to yield *hPDGF-A* tg and wild-type mice. Homozygous *hPDGF-A* tg mice exhibit deformity of the spinal cord (scoliosis) and die within the first few postnatal weeks. Therefore, this study used heterozygous *hPDGF-A* tg mice, which do not have any overt CNS abnormalities. The *hPDGF-A* transgene encodes a 318 bp "short" diffusible alternative-splice isoform of the human *PDGF-A* gene (Pollock and Richardson, 1992), along with a myc epitope tag fused to the carboxy terminus, under the control of the mouse GFAP promoter (Brenner et al., 1994). Hemizygous *hPDGF-A* tg mice exhibit increased expression of PDGF-A in reactive astrocytes during acute (6 weeks) cuprizone induced demyelination (Woodruff et al., 2004). Genotype was determined with PCR of tail DNA, as detailed previously (Fruttiger et al., 2000).

Cuprizone experimental demyelination

Cuprizone ingestion in mice results in a highly reproducible model of corpus callosum demyelination (Matsushima and Morell, 2001; Armstrong et al., 2002). Acute cuprizone administration (6 weeks) is followed by spontaneous remyelination during subsequent weeks on normal chow, whereas following chronic cuprizone administration

(12 weeks) remyelination is limited (Mason et al., 2001, 2004; Armstrong et al., 2006). Male mice at 8 weeks of age were started on 0.2% (w/w) cuprizone diet (finely powdered oxalic bis (cyclohexylidenehydrazide); Aldrich, Milwaukee, WI) mixed into milled chow (Harland Teklad, Certified LM-485 code 7012CM), which was available *ad libitum.* Mice were sacrificed after 3, 6, 9, or 12 weeks on the cuprizone diet, to examine acute and chronic phases of the disease progression. To examine recovery from chronic demyelination, additional mice were fed cuprizone for 12 weeks and then allowed a subsequent 6 weeks on a normal chow diet (Armstrong et al., 2006).

Tissue preparation

Mice were intracardially perfused with 4% paraformaldehyde (Sigma, St. Louis, MO), and then brains were dissected prior to post-fixation in 4% paraformaldehyde at 4^{o}C overnight (Redwine and Armstrong, 1998). Brain tissue was then cryoprotected in 30% sucrose (Sigma, St. Louis, MO) at 4^{o}C overnight, embedded in Tissue Tek OCT (Sakura, Torrance, CA) and stored at –80^{o}C. Coronal sections were cut at 15 μm on a cryostat (Bright Instruments, England) and thaw mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA) for *in situ* hybridization and immunohistochemistry.

Immunohistochemistry

Myelin was immunostained with monoclonal antibody 8-18C5, which recognizes myelin oligodendrocyte glycoprotein (MOG; hybridoma cells provided by Dr. Minetta Gardinier; University of Iowa, Iowa City, IA; Linnington et al., 1984). The hPDGF-A transgene fusion protein was detected by immunostaining for the myc epitope tag with a rabbit polyclonal antibody (MBL, Wobum, MA). MOG and myc immunolabeling were

detected with donkey anti-mouse IgG F(ab')$_2$ conjugated with Cy3 (Jackson Immunoresearch).

***In situ* hybridization**

In situ hybridization and preparation of digoxigenin-labeled riboprobes for proteolipid protein (PLP) and PDGRαR were performed with methods previously detailed (Redwine and Armstrong, 1998; Messersmith et al., 2000; Armstrong et al., 2002). Antisense riboprobes were used to detect mRNA transcripts for PLP (gift from Dr. Lynn Hudson; National Institutes of Health; Hudson et al., 1987), PDGFαR (gift from Dr. Bill Richardson; University College London; Fruttiger et al., 1999), and hPDGF-A (gift from Dr. Marcus Fruttiger; University College London; Fruttiger et al., 1999). Digoxigenin labeled riboprobes were hybridized to coronal brain sections, and digoxigenin was detected with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche, Indianapolis, IN), followed by reaction with NBT/BCIP substrate (DAKO, Carpinteria, CA).

Cell proliferation

Cell proliferation was estimated by incorporation of bromodeoxyuridine (BrdU). At 4 hours and 2 hours prior to perfusion, mice were injected intraperitoneally with 200 mg/kg BrdU (Sigma, St. Louis, MO; as in Redwine and Armstrong, 1998). Following *in situ* hybridization detection for PDGFαR mRNA (see above) sections were processed for immunostaining with a monoclonal anti-BrdU antibody directly conjugated with horseradish peroxidase (mouse monoclonal IgG Fab fragment; Roche, Indianapolis, IN). Peroxidase activity was detected by incubation with 3,3'-diaminobenzidine (DAB; Vector Labs, Burlingame, CA). Cell proliferation was also evaluated by immunostaining

for Ki67 antigen, which is expressed in the nuclei of actively dividing cells but absent at G_0 (Gerdes et al., 1984). Ki67 was recognized with a rat anti-mouse monoclonal antibody (DAKO; Carpinteria, CA) followed by detection with the ABC elite kit using DAB as a substrate (Vector Labs, Burlingame, CA).

Apoptosis

Apoptosis was assessed with a modified TUNEL assay (ApopTag *in situ* apoptosis detection kit; Intergen, Purchase, NY). The 3'-OH DNA ends, generated by DNA fragmentation typically observed with cells undergoing apoptosis, were labeled with digoxigenin-dUTP using terminal deoxynucleotidyl transferase (TdT). The digoxigenin tag was then detected with an anti-digoxigenin antibody conjugated with peroxidase or fluorescein. Peroxidase was detected with DAB substrate (Vector Labs, Burlingame, CA) to yield a dark brown reaction product and nuclei were counterstained with methyl green (Vector Labs, Burlingame, CA). Immunostaining for Olig2 (1:200; Chemicon, Temecula, CA) was used to identify cells at stages throughout the oligodendrocyte lineage (Ligon et al., 2006) in combination with ApopTag using fluorescein detection.

Imaging, quantification, and statistical analysis

All *in situ* hybridization and immunohistochemistry images were acquired with Spot2 software using a digital camera combined with an IX-70 inverted microscope (Olympus, Melville, NY). Images were prepared as panels using Adobe Photoshop (Mountain View, CA). The region analyzed was designated as the corpus callosum, from the midline and extending bilaterally to below the cingulum.

For comparing cell densities, cells expressing PLP mRNA were quantified using unbiased stereological morphometric analysis (Armstrong et al., 2002, 2006; Stereologer System Systems Planning and Analysis, Inc., Alexandria, VA). Using the Stereologer System, the thickness was sampled as part of the definition of each "dissector" volume, so that density measurements reflect cells/mm^3. Unbiased stereology cannot be used appropriately for conditions with relatively low cell densities. Therefore, quantification of cells in the adult mouse corpus callosum expressing PDGFαR mRNA/BrdU, ApopTag, and Ki67 required counting all labeled cells and using the Spot2 camera and software to measure the area sampled (Armstrong et al., 2002, 2006). Without use of the Stereologer System, section thickness could not be sampled in the mounted section, and the density of units is reported as cells/mm^2.

Quantification of corpus callosum myelination was estimated from MOG immunofluorescence, detected with a narrow band pass filter for Cy3 (Chroma Technologies, Brattleboro, VT). Using Metamorph software (Molecular Devices Corp., Downingtown, PA), pixel intensity values were normalized between sections by thresholding to exclude values below the level of immunoreactivity in the dorsal fornix (just inferior to the corpus callosum and not demyelinated by cuprizone). The percent area of the corpus callosum (midline bilaterally to just under the apex of the cingulum) with MOG immunoreactivity above the thresholded level was then used as an estimate of the myelinated area.

Each category analyzed included three or more tissue sections per mouse and three or more mice per condition. Unpaired Student's *t*-test was used to compare between genotypes among non-treated mice. One-way analysis of variance (ANOVA)

with post hoc Tukey's multiple comparison was used to determine significant differences among stages of disease progression or treatment. Significance of a genotype effect across multiple treatment conditions was calculated using two-way ANOVA.

RESULTS

The hPDGF-A transgene is expressed in acute and chronic cuprizone lesions.

Chronic cuprizone demyelination of the corpus callosum is followed by limited remyelination and serves as a reproducible model to test the ability of PDGF-A overexpression to promote remyelination *in vivo*. To determine whether the GFAP promoter maintained *hPDGF-A* transgene expression in chronically demyelinated lesions, we used *in situ* hybridization to detect hPDGF-A mRNA transcripts and immunostaining for the myc epitope tag of the transgene fusion protein (Figure 1). In *hPDGF-A* tg mice, the transgene was most strongly expressed in the corpus callosum during the acute phase of cuprizone treatment. Importantly, expression of the transgene was readily detectable in corpus callosum lesions of mice fed cuprizone for 12 weeks, with reduced signal after removal of cuprizone from the diet to allow recovery after chronic demyelination. At each time point, wild-type mice were examined as controls and lack of signal above background levels demonstrated specificity of the hPDGF-A *in situ* hybridization (data not shown) and the myc immunostaining (Figure 1 A-C).

Overexpression of PDGF-A enhanced remyelination following chronic demyelination.

Overexpression of PDGF-A in *hPDGF-A* tg mice did not alter remyelination following acute demyelination (Woodruff et al., 2004). However, robust remyelination

already occurs following acute but not chronic demyelination models, including cuprizone treatment (Mason et al., 2001; Armstrong et al., 2006). Therefore, we tested the capacity of PDGF-A overexpression to positively influence the limited remyelination that follows chronic cuprizone demyelination in the corpus callosum (Figure 2). Mice of *hPDGF-A* tg and wild-type genotypes were sacrificed at intervals throughout a period of continuous cuprizone ingestion for up to 12 weeks followed by a return to normal chow for a 6-week recovery period. During the cuprizone treatment period, both genotypes resulted in a similar disease progression and at 12 weeks of cuprizone ingestion both genotypes exhibited a similar extent of demyelination. During the recovery period with normal chow for 6 weeks, corpus callosum myelination in the *hPDGF-A* tg mice improved significantly compared to the 12 week cuprizone values (Figure 2B). In contrast, extensive demyelination persisted in wild-type mice during the recovery period (Figure 2A), as observed in C57Bl/6 mice (Armstrong et al., 2006).

PDGF-A overexpression stimulates OP proliferation during acute demyelination but not during chronic demyelination.

PDGF-A signaling can regulate proliferation and amplification of OP cells in response to acute demyelination (Woodruff et al., 2004; Murtie et al., 2005). The OP response in *hPDGF-A* tg and wild-type mice was examined in acute and chronic cuprizone models to determine whether PDGF-A overexpression might improve remyelination in *hPDGF-A* tg mice by increasing the pool of OP cells available for regeneration of oligodendrocytes following chronic demyelination (Figure 3). Prior to the start of cuprizone treatment, wild-type and *hPDGF-A* tg mice had a similar density of OP

cells, which were identified by PDGFαR mRNA transcripts. Also, in non-treated mice, both genotypes exhibited a similar level of OP proliferation, as estimated by BrdU incorporation among PDGFαR+ cells. In response to acute (6 week) cuprizone demyelination, the densities of total OP cells (Figure 3 A,B) and proliferating OP cells (Figure 3 C,D) were significantly increased in *hPDGF-A* tg mice, which was not observed in wild-type mice. Immunostaining for Ki-67 antigen also confirmed increased proliferation in corpus callosum lesions of *hPDGF-A* tg mice compared to wild-type mice during acute cuprizone demyelination (data not shown). Interestingly, this effect of PDGF-A overexpression during the acute disease phase does not continue in the chronic phase. During chronic demyelination (12 week cuprizone) and recovery (12 week cuprizone with 6 week recovery), the OP population and proliferative capacity are similar to non-treated values and are not different between *hPDGF-A* tg and wild-type genotypes (Figure 3 A-D).

PDGF-A overexpression increases oligodendrocyte repopulation of chronic lesions.

PDGFαR expression is mainly associated with the OP stage of the oligodendrocyte lineage but newly generated oligodendrocytes maintain specific responses to PDGF (Barres et al., 1992; Hart et al., 1992). Therefore, oligodendrocyte repopulation of chronic lesions was quantified to further examine the mechanism of improved remyelination with *hPDGF-A* transgene expression (Figure 4). In control mice that were not fed cuprizone, an effect of *hPDGF-A* genotype was evident. The density of oligodendrocytes identified by PLP mRNA *in situ* hybridization was significantly higher in *hPDGF-A* tg mice than in wild-type mice at 8 weeks of age. Cuprizone administration

resulted in significant loss of oligodendrocytes in the corpus callosum at each time point examined indicating that the disease severity was not different in *hPDGF-A* tg versus wild-type mice. In mice of both genotypes, oligodendrocyte densities increase significantly from the 12 week chronic disease level during the 6 week recovery period. However, the *hPDGF-A* tg mice exhibit significantly greater improvement of oligodendrocyte densities during the recovery period compared to the wild-type mice (Figure 4 A, B).

PDGF-A overexpression reduces apoptosis during recovery from chronic demyelination.

Continued cuprizone feeding for 12 weeks causes continued apoptosis of mature oligodendrocytes in C57Bl/6 mice (Mason et al., 2004). Therefore, differences in the extent of cell death were examined as potentially contributing to the improved oligodendrocyte repopulation and remyelination that was observed in *hPDGF-A* tg mice relative to wild-type mice (Figure 5). Relatively low levels of TUNEL positive cells were present in 8-week old mice of either genotype in the absence of cuprizone treatment. During the acute phase of cuprizone treatment, *i.e.* up to 6 weeks, the peak of oligodendrocyte apoptosis occurs around 3 weeks (data not shown; Mason et al., 2000) so this time point was chosen for comparing acute and chronic stages. As expected from the similar oligodendrocyte loss in *hPDGF-A* tg and wild-type mice with cuprizone ingestion (Figure 4), the extent of apoptosis was similar between these genotypes at 3 weeks (acute) and at 12 weeks (chronic) of cuprizone treatment (Figure 5 A, B). Surprisingly, following 12 weeks of cuprizone administration there was continued apoptosis during the

6-week recovery period on a normal chow diet. During the recovery period, significantly fewer apoptotic cells were detected in the corpus callosum of *hPDGF-A* tg mice relative to wild-type mice (Figure 5 A, B). Apoptotic cells included those within rows of cells aligned longitudinally between axons, a characteristic of interfascicular oligodendrocytes (Figure 5C), and cells that were double labeled for Olig2 immunostaining (Figure 5D-F) to identify immature through mature stages of the oligodendrocyte lineage. Therefore, PDGF-A may promote cell survival during recovery from chronic demyelination, which correlates well with a previously demonstrated role for PDGF-A in preventing oligodendrocyte apoptosis during myelination in development (Barres et al., 1992).

DISCUSSION

Remyelination of MS lesions becomes limited following repeated or prolonged episodes of demyelination. Factors influencing this progression to a chronic disease state are important to identify because denuded axons have impaired neurotransmission and increased vulnerability to damage. Studies using cuprizone to induce chronic demyelination have demonstrated limited remyelination that is associated with depletion of OP cells and inhibition of OP differentiation in lesions (Mason et al., 2004; Armstrong et al., 2006). Our current results show that apoptosis continues after removal of cuprizone from the diet and is an additional factor contributing to the limited remyelination observed following chronic demyelination. Furthermore, PDGF-A overexpression reduces apoptosis following chronic demyelination, increases oligodendrocyte regeneration, and significantly promotes remyelination of corpus callosum lesions.

The chronic cuprizone model of demyelination is advantageous for evaluating potential improvements in remyelination. Acute demyelination models with robust OP amplification and complete remyelination are well suited for examining how successful remyelination can be accomplished. Conversely, strategies to improve remyelination can be difficult to test in the context of extensive spontaneous remyelination. Indeed, analysis of *hPDGF-A* tg mice using acute toxin-induced demyelination did not detect an effect on remyelination (Woodruff et al., 2004). The chronic cuprizone model in C57Bl/6 mice results in apoptosis of regenerated oligodendrocytes upon maturation which leads to chronic demyelination with eventual depletion of the OP population and limited remyelination even after removal of cuprizone from the diet (Mason et al., 2001; Armstrong et al., 2006). In the current study, this chronic disease scenario revealed significant improvement in the extent of remyelination in *hPDGF-A* tg mice (Figure 2).

Expression of the *hPDGF-A* transgene under control of the astrocyte GFAP promoter is especially appropriate for analyses in the context of demyelination. Endogenous PDGF-A is synthesized in reactive astrocytes associated with demyelinated lesions (Redwine and Armstrong et al., 1998; Hinks and Franklin, 1999; Frost et al., 2003). In *hPDGF-A* tg mice, the GFAP promoter upregulates *hPDGF-A* expression in correlation with an astroglial response to demyelination, as observed using *in situ* hybridization for hPDGF-A mRNA transcripts (Figure1; Woodruff et al., 2004) or the myc tag to localize the transgene product in lesion areas (Figure 1). Overexpression of PDGF in the adult CNS can induce abnormal hyperplasias and tumor formation in immature cells of the corpus callosum or the subventricular zone (Assanah et al., 2006; Jackson et al., 2006). However, regulated expression of the *hPDGF-A* transgene from the

GFAP promoter in heterozygous mice did not result in hyperplasias or tumors in the current study of mice up to 26 weeks of age examined using BrdU incorporation (Figure 3) or immunostaining for Ki-67 antigen (data not shown). Driving PDGF-A overexpression in lesions via astrocytes may also facilitate signaling with appropriate extracellular matrix molecule interactions, such as tenascin-C modulation of PDGF-A induced OP proliferation and survival (Garwood et al., 2004).

We initially predicted that PDGF-A overexpression in *hPDGF-A* tg mice would improve remyelination by acting as a OP mitogen to counter the depletion of OPs during chronic demyelination. Indeed, OP density increased dramatically during acute demyelination in *hPDGF-A* tg mice (Figure 3), consistent with a previous report (Woodruff et al., 2004). This OP response in *hPDGF-A* tg mice is even more notable given the poor OP response in the wild-type littermates (Figure 3), which indicates that this genetic background may be less favorable for OP proliferation than other lines we have examined (Armstrong et al., 2006). Surprisingly, the increased OP response to acute demyelination in *hPDGF-A* tg mice did not translate to an elevation of OP cell density or proliferation during the chronic disease stage. OP densities were similar after chronic cuprizone demyelination in *hPDGF-A* tg and wild-type (Figure 3), and these values were similar to those measured after chronic cuprizone treatment in C57Bl/6 mice (Armstrong et al., 2006). Thus, improved remyelination of chronic lesions in *hPDGF-A* tg mice is unlikely to result from stimulating OP proliferation.

PDGF-A can also act as a survival factor during oligodendrocyte development (Barres et al., 1992; Gard et al., 1995; Grinspan and Franceschini, 1995). Relatively low concentrations of PDGF-A can promote survival as developing OP cells exit the cell

cycle and generate new oligodendrocytes prior to down regulation of PDGFαR expression (Hart et al., 1992; Barres et al., 1992). During remyelination, newly generated oligodendrocytes may be particularly vulnerable prior to establishing effective interactions with axons that can provide survival signals for myelinating oligodendrocytes (Trapp et al., 1997; Barres and Raff, 1999). An effect of PDGF-A in promoting survival of newly generated oligodendrocytes (Barres et al., 1992) is consistent with our results in *hPDGF-A* tg mice during the chronic stages of cuprizone demyelination and recovery. OP cells continue to generate new oligodendrocytes during the course of chronic cuprizone treatment (Mason et al., 2001; Armstrong et al., 2006). Upon maturation, the newly generated cells become susceptible to cuprizone toxicity and undergo apoptosis. After cuprizone is removed from the diet, we show that apoptosis continues in the chronic lesion environment (Figure 5). During this recovery period, the reduced level of apoptosis in *hPDGF-A* tg mice may allow survival of a greater number of newly generated oligodendrocytes (Figure 4) and result in improved remyelination (Figure 2) compared to wild-type mice.

This interpretation of PDGF-A as a survival factor for newly generated oligodendrocytes in chonically demyelinated lesions has important implications for promoting remyelination in demyelinating diseases. Multiple mechanisms of cell death are associated with active demyelination, such as immune and inflammatory responses and glutamate excitotoxicity. The current results (Figure 5) demonstrate additional cell death that is not associated with active demyelination but instead occurs during failed attempts at oligodendrocyte regeneration. Following acute cuprizone demyelination, apoptosis is extremely rare several weeks after returning mice to a normal diet

(Armstrong et al., 2002; Mason et al., 2004). In contrast, following chronic cuprizone demyelination we found that apoptosis continues after removal of cuprizone from the diet. Thus, the environment of chronically demyelinated lesions may not provide adequate support for survival of newly generated oligodendrocytes and may contribute to the limited capacity for remyelination of chronic lesions. PDGF-A overexpression appears to improve cell survival during the period for recovery following chronic demyelination. An effect of PDGF-A on survival of newly generated oligodendrocytes rather than mature cells is indicated by the lack of protection of mature myelinating oligodendrocytes during acute cuprizone toxicity in *hPDGF-A* tg mice (3 wk cup: Figure 4). These results in *hPDGF-A* tg mice clearly differ from effects of insulin-like growth factor 1 or leukemia inhibitory factor, which each prevent loss of mature myelinating oligodendrocytes during active demyelination induced by acute cuprizone ingestion (Mason et al., 2001; Emery et al., 2006).

Attenuation of detrimental effects of the chronic lesion environment has revealed a remarkable capacity of endogenous OP cells to remyelinate chronic lesions in animal models. In addition to the current findings of improved remyelination with decreased apoptosis in *hPDGF-A* tg mice, a similar study demonstrated almost complete remyelination following chronic cuprizone demyelination in *fibroblast growth factor 2 (FGF2)* null mice (Armstrong et al., 2006). FGF2 is upregulated in demyelinated lesions and can inhibit OP differentiation into oligodendrocytes (Armstrong et al., 2002, 2006; Murtie et al., 2005). Following chronic demyelination, the depleted OP population more effectively generated remyelinating oligodendrocytes in the absence of FGF2 (Armstrong et al., 2006). Thus the ability of endogenous cells to remyelinate chronic lesions may be

controlled not only by the number of available OP cells but also by the permissiveness of the environment for those OP cells to differentiate and survive to efficiently generate remyelinating oligodendrocytes.

Immature oligodendrocyte lineage cells can persist in MS lesions (Wolswijk, 1998; Maeda et al., 2001; Chang et al., 2002; Wilson et al., 2006). However, MS lesions may have increased expression of signals that inhibit differentiation (John et al., 2002; Back et al., 2005) or variable expression of molecules that support newly generated oligodendrocytes to survive and myelinate viable axons (Viehover et al., 2001; Vanderlocht et al., 2006). Such conditions may have detrimental effects on endogenous cells and may also compromise the potential of transplanted cells to remyelinate throughout large areas of demyelination (Keirstead et al., 2005). Therefore, treatments for chronic demyelinating diseases may require attenuation of active demyelination as well as alteration of the lesion environment to optimize remyelination.

FIGURE 1

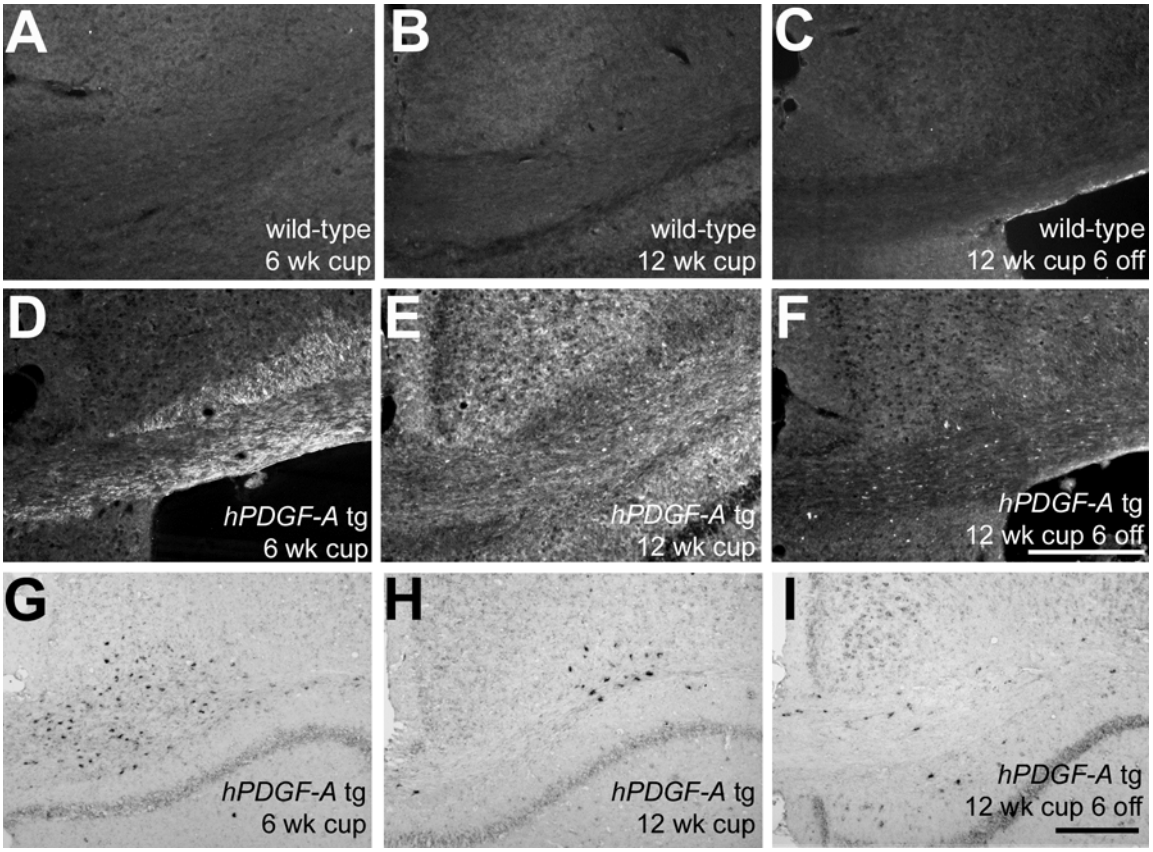

Figure 1. ***Detection of hPDGF-A transgene expression in cuprizone demyelinated corpus callosum***. **A-F:** Immunofluorescence detection of myc epitope tag of *hPDGF-A* transgene fusion protein in coronal sections of corpus callosum in wild-type (**A-C**) and *hPDGF-A* transgenic (*hPDGF-A* tg) mice (**D-F**). **G-I:** *In situ* hybridization to detect hPDGF-A transgene mRNA transcripts in *hPDGF-A* tg mice. The midline is along the left border in each image. Mice were fed chow with 0.2% cuprizone and then analyzed following acute cuprizone demyelination (**A, D, G**; 6 wk cup), chronic cuprizone demyelination (**B, E, H**; 12 wk cup), or chronic demyelination followed by a 6 week recovery period on normal chow (**C, F, I**; 12 wk cup 6 off). Scale bar (250 μm) for A-F as shown in F and for G-I as shown in I.

FIGURE 2

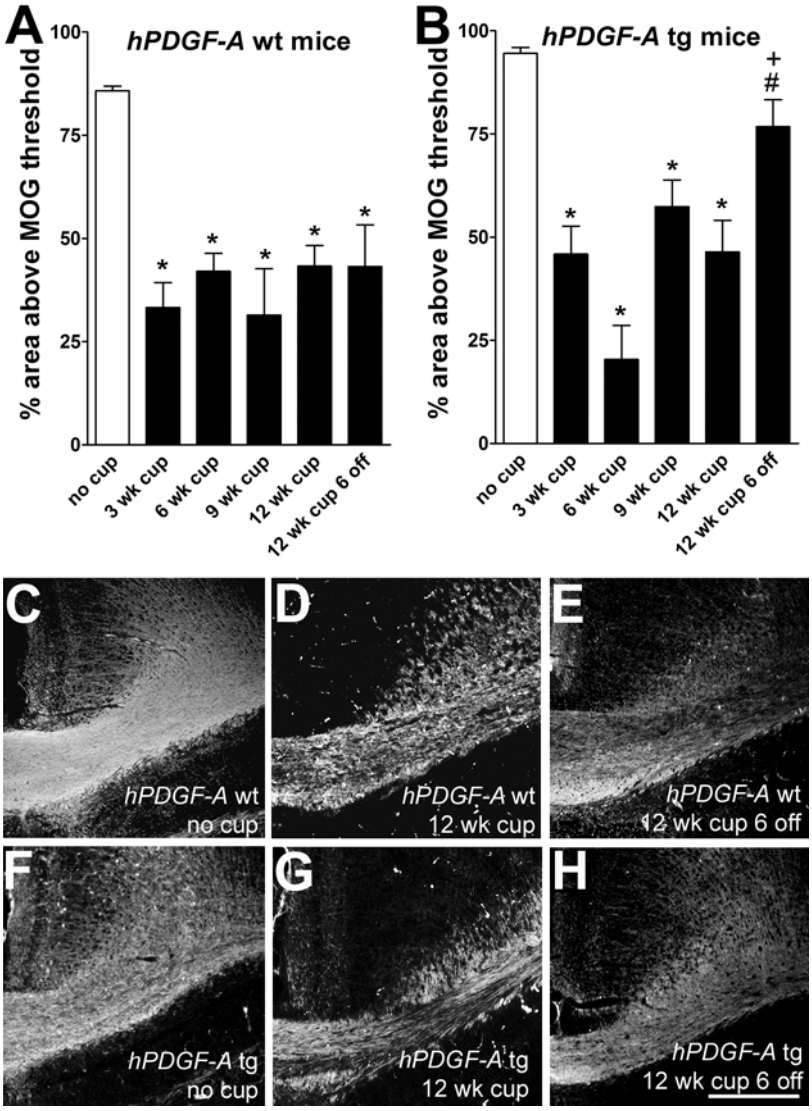

Figure 2. ***PDGF-A overexpression promotes spontaneous remyelination after chronic demyelination.*** **A, B:** Quantification of corpus callosum myelination estimated by immunofluorescence for myelin oligodendrocyte glycoprotein (MOG) in *hPDGF-A* wild-type (wt) mice (**A**) and transgenic (tg) mice (**B**). Black bars indicate perfusion after 3, 6, 9, or 12 weeks of continuous 0.2% cuprizone feeding, or 12 weeks of cuprizone followed by 6 weeks on normal chow. White bars indicate no cuprizone treatment. Pixel intensity values were normalized between tissue sections by thresholding to exclude values below

the level of immunoreactivity in the dorsal fornix, which is not demyelinated by cuprizone. At least three sections were quantified per mouse from at least three mice per condition. In mice of both genotypes, significant demyelination occurs with cuprizone treatment (***** $p < 0.05$ compared to no cup; one-way ANOVA within each genotype). The *hPDGF-A* tg mice show significant remyelination of the corpus callosum during the recovery period on normal chow relative to the chronic cuprizone demyelination (**#** $p < 0.05$ for *hPDGF-A* tg 12 wk cup 6 off compared to *hPDGF-A* tg 12 wk cup; one-way ANOVA within genotype). Further, remyelination in the *hPDGF-A* tg mice is significantly increased from the *hPDGF-A* wt mice (**+** $p < 0.05$ for *hPDGF-A* tg 12 wk cup 6 wk off compared to *hPDGF-A* wt 12 wk cup 6 wk off; two-way ANOVA for genotype and treatment). With this pattern during the recovery period, the values for *hPDGF-A* tg mice have returned to within normal levels ($p > 0.05$ for 12 wk cup 6 off compared to no cup; one-way ANOVA within genotype) while *hPDGF-A* wt values remain significantly below normal levels ($p < 0.001$ for 12 wk cup 6 off compared to no cup; one-way ANOVA within genotype). **C-H:** Representative images of MOG immunostaining of coronal sections through the corpus callosum, with midline along the left border in each image. **C:** *hPDGF-A* wt control mouse maintained on normal chow, without cuprizone treatment. **D:** *hPDGF-A* wt mouse fed cuprizone continuously for 12 weeks. **E:** *hPDGF-A* wt mouse fed cuprizone continuously for 12 weeks, followed by 6 weeks of recovery. **F:** *hPDGF-A* tg control mouse maintained on normal chow, without cuprizone treatment. **G:** *hPDGF-A* tg mouse fed cuprizone continuously for 12 weeks. **H:** *hPDGF-A* tg mouse fed cuprizone continuously for 12 weeks, followed by 6 weeks of recovery. Scale bar for C-H shown in H is 250 μm.

FIGURE 3

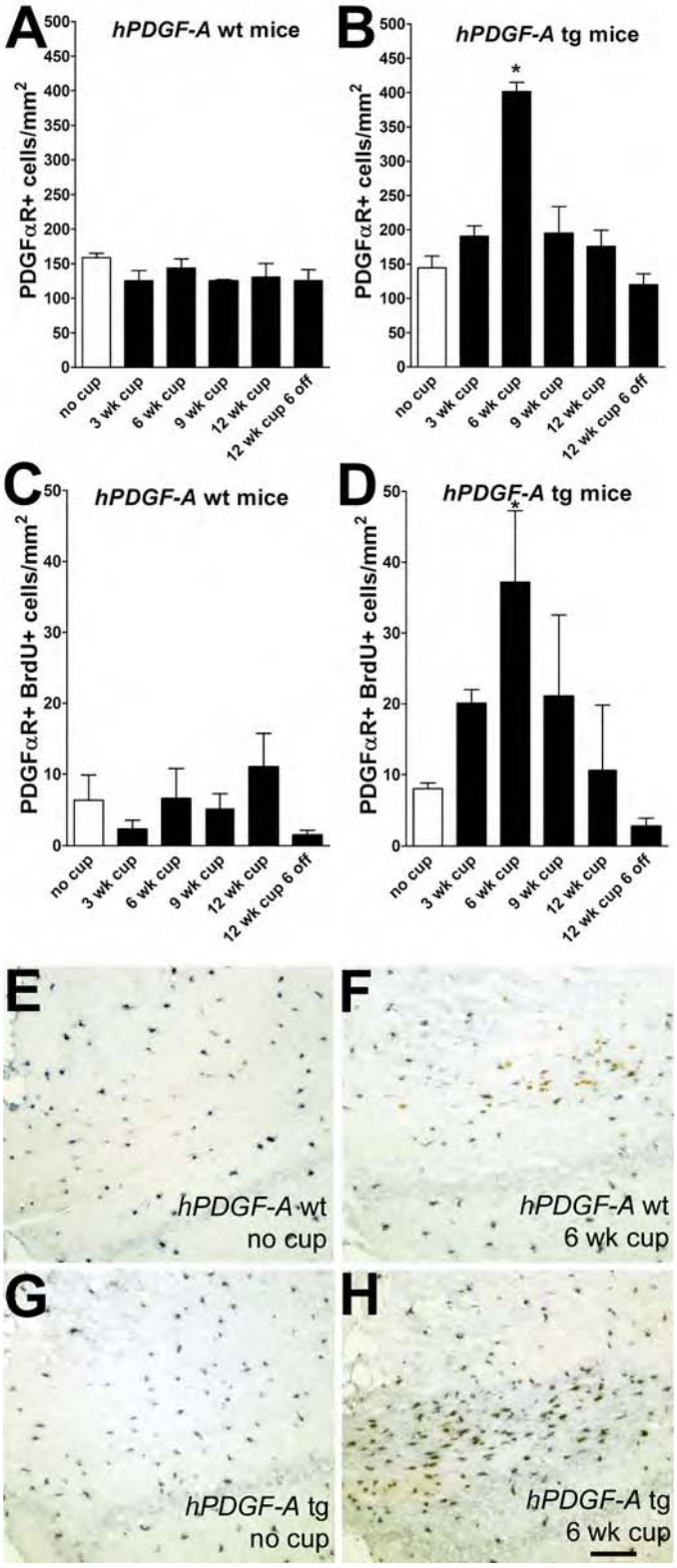
A
hPDGF-A wt mice
PDGFαR+ cells/mm2
500
450
400
350
300
250
200
150
100
50
0
no cup
3 wk cup
6 wk cup
9 wk cup
12 wk cup
12 wk cup 6 off
B
hPDGF-A tg mice
*
PDGFαR+ cells/mm2
C
hPDGF-A wt mice
PDGFαR+ BrdU+ cells/mm2
50
40
30
20
10
0
D
hPDGF-A tg mice
*
PDGFαR+ BrdU+ cells/mm2
E
hPDGF-A wt
no cup
F
hPDGF-A wt
6 wk cup
G
hPDGF-A tg
no cup
H
hPDGF-A tg
6 wk cup

Figure 3. ***PDGF-A overexpression stimulates OP population only during acute stage of cuprizone demyelination.*** **A-D:** Quantification of the oligodendrocyte progenitor (OP) response in the corpus callosum of *hPDGF-A* wild type (wt; A, C) and transgenic (tg; B, D) mice. Black bars indicate perfusion after 3, 6, 9, or 12 weeks of continuous 0.2% cuprizone feeding, or 12 weeks of cuprizone followed by 6 weeks on normal chow. White bars indicate no cuprizone treatment. At least three sections were quantified per mouse from at least three mice per condition. **A, B:** Density of the total OP population, identified by PDGFαR mRNA expression. **C, D:** Density of proliferating OP cells identified by PDGFαR mRNA and incorporation of BrdU during a 4 hour terminal pulse. Prior to the start of cuprizone treatment, *hPDGF-A* mice of both genotypes have similar densities of total OP cells (A, B) and similar extents of proliferation among the OP pool (C, D). After 6 weeks of cuprizone treatment in *hPDGF-A* tg mice, significant increases are observed in the total OP cell density (B, ***** $p < 0.001$) and the extent of OP proliferation (D, ***** $p < 0.001$) compared to *hPDGF-A* tg mice without cuprizone treatment. **E-H**: Representative coronal sections showing PDGFαR mRNA *in situ* hybridization (blue/black cytoplasm) and BrdU incorporation (brown nuclei) in the corpus callosum, with midline along the left border in each image. **E:** *hPDGF-A* wt mouse maintained on normal chow, without cuprizone treatment. **F:** *hPDGF-A* wt mouse fed cuprizone continuously for 6 weeks. **G:** *hPDGF-A* tg mouse maintained on normal chow, without cuprizone treatment. **H:** *hPDGF-A* tg mouse fed cuprizone continuously for 6 weeks. Scale bar for E-H shown in H is 250 μm.

FIGURE 4

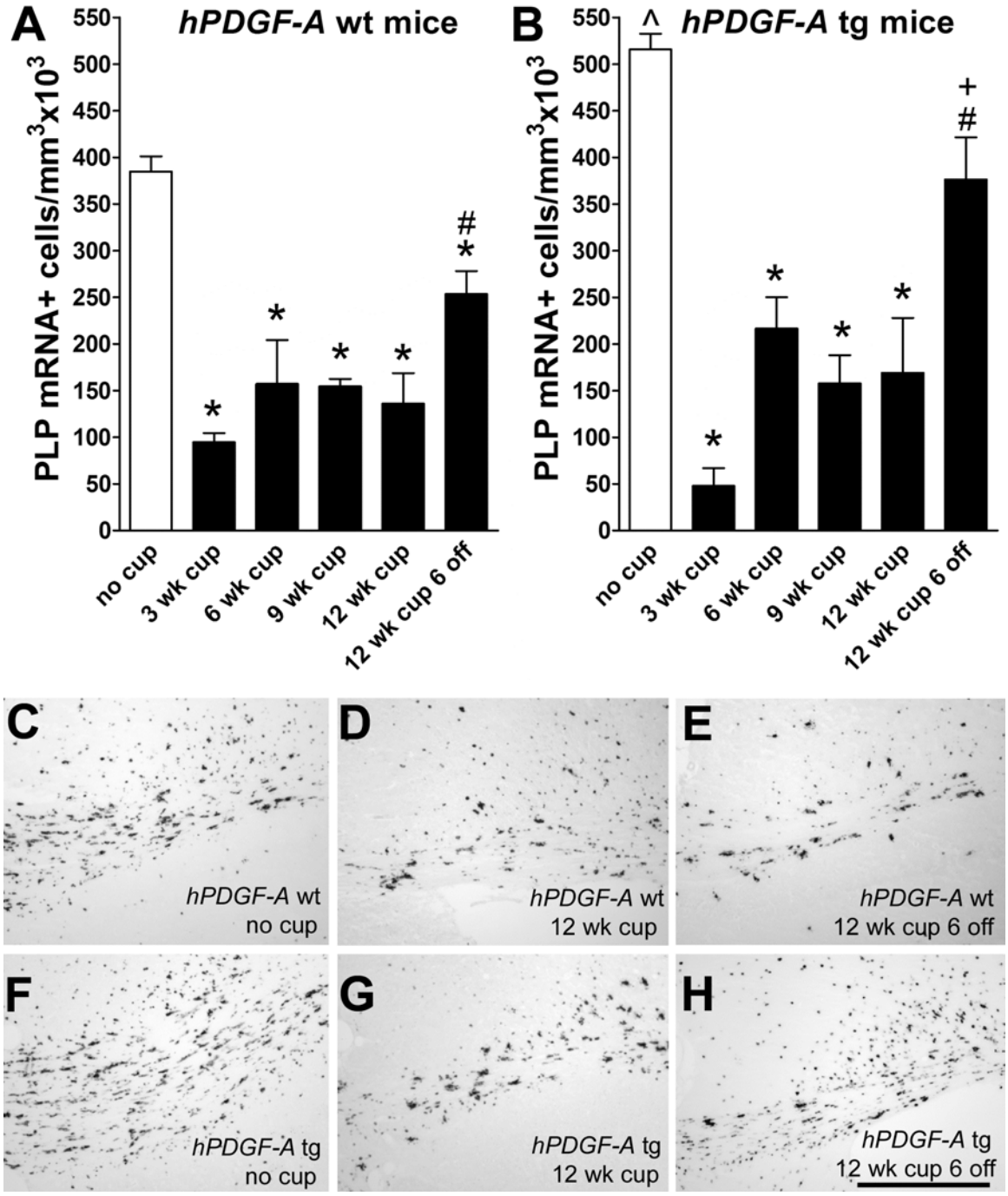

Figure 4. ***PDGF-A overexpression enhances oligodendrocyte repopulation after chronic cuprizone treatment.*** **A, B:** Quantification of the density of oligodendrocytes,

identified by *in situ* hybridization for PLP mRNA, in the corpus callosum of *hPDGF-A* wild type (wt; A) and transgenic (tg; B) mice. Black bars indicate perfusion after 3, 6, 9, or 12 weeks of continuous 0.2% cuprizone feeding, or 12 weeks of cuprizone followed by 6 weeks on normal chow. White bars indicate no cuprizone treatment. At least three sections were quantified per mouse from at least three mice per condition. Prior to the start of cuprizone treatment, *hPDGF-A* tg mice have a higher density of oligodendrocytes than *hPDGF-A* wt mice at 8 weeks of age (^ $p = 0.0009$; Student's t-test between genotypes without treatment). During cuprizone treatment of both *hPDGF-A* wt and tg mice, the density of oligodendrocytes is significantly decreased from normal levels (***** $p < 0.0001$ compared to no cup; one-way ANOVA within each genotype). Both genotypes show significant oligodendroglial repopulation of the corpus callosum during the recovery period (**#** $p < 0.05$ for 12 wk cup 6 off compared to 12 wk cup; one-way ANOVA within genotype). However, the oligodendroglial repopulation in the *hPDGF-A* tg mice is significantly increased from the *hPDGF-A* wt mice (**+** $p < 0.05$ for *hPDGF-A* tg 12 wk cup 6 wk off compared to *hPDGF-A* wt 12 wk cup 6 wk off; two-way ANOVA for genotype and treatment). With this pattern during the recovery period, the values for *hPDGF-A* tg mice have returned to within normal levels ($p > 0.05$ for 12 wk cup 6 off compared to no cup; one-way ANOVA within genotype) while *hPDGF-A* wt values remain significantly below normal levels (* $p < 0.05$ for 12 wk cup 6 off compared to no cup; one-way ANOVA within genotype). **C**-**H**: Representative coronal sections showing PLP mRNA *in situ* hybridization in the corpus callosum, with midline along the left border in each panel. **C:** *hPDGF-A* wt control mouse maintained on normal chow, without cuprizone treatment. **D:** *hPDGF-A* wt mouse fed cuprizone continuously for 12

weeks. **E:** *hPDGF-A* wt mouse fed cuprizone continuously for 12 weeks, followed by 6 weeks of recovery. **F:** *hPDGF-A* tg mouse maintained on normal chow, without cuprizone treatment. **G:** *hPDGF-A* tg mouse fed cuprizone continuously for 12 weeks. **H:** *hPDGF-A* tg mouse fed cuprizone continuously for 12 weeks, followed by 6 weeks of recovery. Scale bar for C-H shown in H is 250 μm.

FIGURE 5

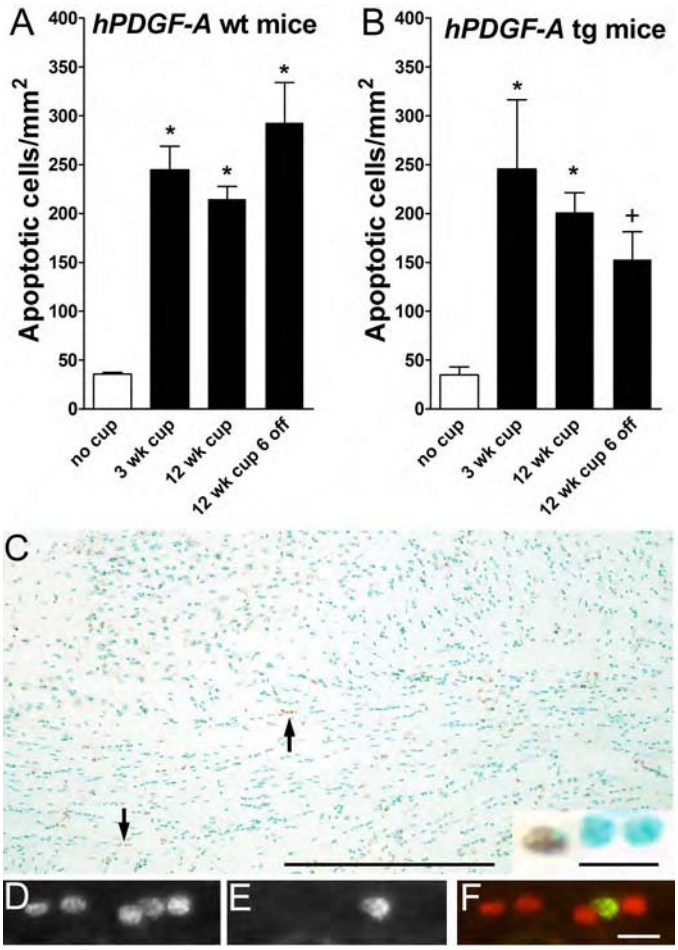

Figure 5. ***PDGF-A overexpression decreased apoptosis in the corpus callosum after chronic cuprizone treatment.*** **A, B:** Quantification of the density of apoptotic cells, identified by modified TUNEL assay, in the corpus callosum of *hPDGF-A* wild type (wt; A) and transgenic (tg; B) mice. Black bars indicate perfusion after 3 or 12 weeks of continuous 0.2% cuprizone feeding, or 12 weeks of cuprizone followed by 6 weeks on normal chow. White bars indicate no cuprizone treatment. At least three sections were quantified per mouse from at least three mice per condition. During cuprizone treatment

of both *hPDGF-A* wt and tg mice, the density of apoptotic cells is significantly increased from normal levels (***** $p < 0.05$ compared to no cup; one-way ANOVA within each genotype). Elevated apoptosis values continued after removal of cuprizone from the diet of *hPDGF-A* wt mice (***** $p < 0.05$ compared to no cup). While apoptosis remains elevated somewhat during recovery in *hPDGF-A* tg mice, the values are no longer significantly above those in non-treated *hPDGF-A* tg mice. Further, apoptosis in the *hPDGF-A* tg mice is significantly decreased from the *hPDGF-A* wt mice (**+** $p < 0.05$ for *hPDGF-A* tg 12 wk cup 6 wk off compared to *hPDGF-A* wt 12 wk cup 6 wk off; two-way ANOVA for genotype and treatment). **C:** Representative coronal section showing the corpus callosum (midline along left border) in an *hPDGF-A* wt mouse treated with cuprizone for 12 weeks followed by a 6 week period for recovery. TUNEL (brown) signal is present in individual cells distributed throughout the corpus callosum and in groups of cells aligned as is characteristic of interfascicular oligodendrocytes (arrows and inset). Nuclei stained with methyl green. Scale bars = 250 μm and 10 μm. **D-F:** Immunostaining for Olig2 (D, and red in F) to identify oligodendrocyte lineage cells in combination with TUNEL (E, and green in F) in a coronal section of corpus callosum in an *hPDGF-A* wt mouse treated with cuprizone for 12 weeks followed by a 6-week period for recovery. Scale bar for D-F shown in F is 10 μm.

CHAPTER 4

Conclusion

Summary of Findings

Differences in the oligodendrocyte progenitor (OP) response following acute and chronic demyelination

In these studies we were interested in the OP response to acute and chronic demyelination. The generally accepted paradigm has been that following an acute episode of demyelination the proliferation of OPs generally precedes oligodendrocyte regeneration and remyelination (Carroll et al., 1998; Keirstead et al., 1998; Redwine and Armstrong, 1998; Reynolds et al., 2001; Watanabe et al., 2002), but does the same OP response occur after chronic demyelination? In the first study we set out to characterize increased expression of the transcription factor Myt1, as well as the phenotype of cells that express Myt1, in acute demyelination. Previous studies have shown that Myt1 expression is increased in oligodendrocyte lineage cells in developmental myelination (Armstrong et al., 1995), and after spinal cord injury (Wrathall and Hudson, 1998). *In vitro* Myt1 was found to positively influence OP proliferation and differentiation, as well as myelin gene transcription (Nielsen et al., 2004). We found that during acute demyelination Myt1 mRNA transcripts and protein were significantly increased. The observed increase for Myt1 mRNA and nuclear localized Myt1 protein was similar, thus

indicative of transcripts being translated into functional proteins. Characterization of cell phenotypes that expressed nuclear Myt1 elucidated that the majority of OPs expressed Myt1 (Chapter 2, Figure 4). Only a small number of terminally differentiated oligodendrocytes expressed Myt1 (Chapter 2, Figure 5), it is likely that as OPs differentiated into oligodendrocytes and began to express myelin genes that Myt1 was downregulated similar to what occurs in development (Armstrong et al., 1995). Not all Myt1 expression could be linked to oligodendrocyte lineage cells and the next largest population of cells that expressed Myt1 after OPs consisted of neural stem cells (NSCs; Chapter 2, Figure 6). The likely continuum is that Myt1 begins to be expressed as NSCs proceed toward an oligodendrocyte cell lineage fate, these Myt1 positive NSCs go on to become OPs, as Myt1 is expressed by the majority of OPs, and soon after terminal differentiation into oligodendrocytes Myt1 is finally downregulated. During active demyelination and early remyelination Myt1+ cells and proliferating Myt1+ BrdU+ cells were found to be increased (Chapter 2, Figure 2), and increased Myt1+ cells were noted to occur specifically within lesioned white matter (Chapter 2, Figure 3). Likely that within the lesion environment the production of some undetermined factor is influencing Myt1 expression for the promotion of oligodendrocyte regeneration and remyelination. Further, our studies looked at Myt1 expression in human tissue from MS patients (Chapter 2, Figure 10 and Table I). In MS cases the highest density of Myt1 expression was found in early remyelinating lesions, consistent with our findings in the MHV-A59 and cuprizone models of acute demyelination with spontaneous remyelination. These findings, coupled with previous developmental and *in vitro* work, implicate Myt1 in the generation of oligodendrocyte lineage cells after acute demyelination. After acute

demyelination we have shown that the response of OPs to generate new oligodendrocytes is positively regulated by the transcription factor Myt1. With transcription factors acting as downstream effectors to induce gene transcription after ligand binding of receptors induced signaling, such as the binding of growth factors to their cognate receptors.

Our next study was into the effect that overexpression of platelet-derived growth factor A (PDGF-A) would have after chronic demyelination. PDGF-A is a potent mitogen of OPs *in vitro* and *in vivo* (Besnard et al., 1987; Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988; Calver et al., 1998; Allamargot et al., 2001; Murtie et al., 2005), and promotes survival of newly differentiated oligodendrocytes (Barres et al., 1992, 1993). Previous studies with chronic cuprizone treatment induced demyelination have shown that the OP response is blunted or nonexistent (Mason et al., 2001, 2004; Armstrong et al., 2006). With PDGF-A having been previously found to induce OP proliferation in acute demyelination (Allamargot et al., 2001; Woodruff et al., 2004), it seemed likely to expect that increased expression of PDGF-A may have a similar effect on OP proliferation after chronic demyelination. With the overexpression of PDGF-A we observed an increase in OP density and proliferating OPs during the acute phase of cuprizone induced demyelination, similar to the previously cited study by Woodruff et al. (2004). However, in the chronic phase of demyelination the overexpression of PDGF-A had no effect on the density of OPs or proliferating OPs (Chapter 3, Figure 3). Thus, the OP response, or more correctly the lack of OP amplification, after chronic demyelination differs from the response found in acute demyelination. More strikingly, after chronic demyelination and allowing for recovery the overexpression of PDGF-A resulted in increased remyelination (Chapter 3, Figure 2), increased oligodendrocyte regeneration

(Chapter 3, Figure 4), and decreased apoptosis (Chapter 3, Figure 5). The most likely scenario is that the overexpression of PDGF-A increased survival of newly differentiated oligodendrocytes, which then went on to remyelinate chronically demyelinated lesions. The findings of these two studies further substantiate previous findings of OP proliferation after acute demyelination, here mediated by increased expression of the transcription factor Myt1. However, after chronic demyelination a blunted OP response was evident, even with overexpression of the potent OP mitogen PDGF-A, but is likely that signaling through a PDGFR enhanced survival of newly differentiated oligodendrocytes and resulted in enhanced oligodendrocyte cell regeneration and remyelination. These two mechanisms are interconnected as ligand induced signaling through a receptor activates signaling cascades, which are known to effect transcription factors and ultimately gene transcription.

Contribution of Present Findings

The basic dogma of remyelination has been that for remyelination to occur the pool of OPs must first proliferate with a large amplification of OP cell density, and then differentiate into new oligodendrocytes, and finally these newly generated oligodendrocytes must survive. From this scenario it seems intuitive to enhance the number of immature oligodendrocyte lineage cells to generate new mature oligodendrocytes, however it also is imperative that the newly generated oligodendrocytes survive, which we show can be influenced by trophic factors such as PDGF-A. In that during development upwards of 50% of newly differentiated oligodendrocytes are lost to cell death (Barres et al., 1992, 1993), it is likely that

following OP expansion during acute demyelination a large percentage of newly differentiated oligodendrocytes may be lost. However, after chronic demyelination we have shown that from a depleted pool of OPs the survival of a limited number of new oligodendrocytes is robust enough to enhance remyelination. The study presented here with the overexpression of PDGF-A supports the concept that oligodendrocyte survival from a limited population of OPs can result in significant remyelination. In our studies we had an amplification of OPs in acute cuprizone and MHV-A59 induced demyelination, but in chronic demyelinated lesions, where there is a depleted pool of OPs, an amplification of OPs was not observed. This limited pool of endogenous OPs following chronic demyelination does not undergo major amplification, but if the limited number of OPs can be induced to differentiate and survive, as we found in our study, then an increase in oligodendrocyte density would be observed, and more importantly lead to extensive remyelination. Thus, our study of chronic demyelination with a limited OP response but extensive remyelination confounds the recognized remyelination dogma of the necessity for OP expansion prior to remyelination. Even after allowing six weeks for recovery following the cessation of cuprizone treatment wt mice had significantly increased densities of apoptotic cells within lesions, in essence the environment of a chronic lesion appears nonconducive to oligodendrocyte regeneration and remyelination without some form of manipulation. This was demonstrated in studies where following chronic demyelination either the transplantation of OPs (Mason et al., 2004), or enhancing oligodendrocyte differentiation using fibroblast growth factor 2 (FGF2) knock out mice, which promotes OP differentiation into oligodendrocytes (Armstrong et al., 2006), were able to positively influence oligodendrocyte regeneration and remyelination

after chronic demyelination. Now we show that PDGF-A overexpression promotes oligodendrocyte regeneration, and even more importantly remyelination of chronically demyelinated brain.

With PDGF-A known to induce tumorigenesis an important question is would the overexpression of PDGF-A induce tumor formation? In hPDGF-A tg mice we did not observe any tumors visually or with immunostaining with Ki67, a surrogate marker that labels actively dividing cells. Importantly, the concentration of PDGF-A which was found to induce survival of newly differentiated oligodendrocytes *in vitro* was 200 fold lower than the concentration needed for OP proliferation (Barres et al., 1992, 1993). Thus, a limited increase of PDGF-A may be potent enough to be utilized for remyelination without worry of tumor formation.

Our findings elucidate that increased expression of Myt1 is common to demyelination-remyelination. As two distinctly different models of demyelination, and even more importantly in human MS tissue, the expression of Myt1 is observed within or near lesions at a time when oligodendrocyte regeneration and remyelination is expected to occur. With Myt1 being expressed from NSCs, likely committed toward an oligodendrocyte lineage cell fate, through the oligodendrocyte lineage until oligodendrocyte cells begin transcription of myelin genes. Thus, Myt1 seems a likely candidate to positively influence regeneration of oligodendrocytes and ultimately remyelination after demyelination.

Future Experiments

Determining mechanisms to enhance remyelination could potentially restore rapid axonal conduction and prevent axonal transection in diseases such as MS and following spinal cord injury, as well as in neurodegenerative disorders such as adrenoleukodystrophy and Canavan's disease. One area to be addressed is to determine the cofactors that associate with Myt1, since increased expression of Myt1 is already apparent in areas undergoing demyelination/remyelination, and therefore the expression of Myt1 does not need to be enhanced. However, the other molecules that associate with Myt1 to form a functional transcriptome may be the rate limiting component(s). Work done *in vitro* using full length Myt1 and the dominant negative 4FMyt1 surprisingly had similar effects on OP differentiation (Neilsen et al., 2004). Nielsen et al. (2004) theorized as to why the full length and dominant-negative Myt1 had similar results and attributed this to excess free Myt1 binding the rate limiting cofactors. Essentially, at different time points those cofactors that associate with Myt1 could induce proliferation, while other cofactors may promote differentiation of OPs into oligodendrocytes. Sin3b has been identified as one of the molecules that associates with Myt1 (Romm et al., 2005), and as there are different known isoforms of Sin3b. With one isoform of Sin3b affecting histone acteylase and another isoform affecting histone deacetylase. Thus, whichever isoform is in abundance to form a transcriptome with Myt1 may yield differential effects on activation or repression of transcription. The generation of a Myt1 knockout or transgenic mouse line could offer further insight into the role Myt1 has in developmental myelination and remyelination. The Myt1 knock out may be similar to what was found with PDGFαR and PDGF-A knock outs, where the loss of those particular genes were

either embryonic lethal or soon after birth the animal died, and may thus necessitate the design of a Myt1 conditional knock out mouse.

Much of the work done on oligodendrocyte lineage cells has elucidated that different factors are associated with either proliferation or differentiation. The potent mitogens PDGF-A and FGF2 are commonly combined *in vitro* to enhance OP proliferation, while to induce OP differentiation high insulin and thyroid hormone are utilized. What about different factors in the survival of oligodendrocyte lineage cells? Once OPs differentiate into oligodendrocytes the survival of these cells is paramount for myelination to take place. In our study with the overexpression of PDGF-A there was decreased apoptosis, which correlated with an increased density of terminally differentiated oligodendrocytes. This increased density of oligodendrocytes in *hPDGF-A* tg mice further lead to enhanced remyelination after chronic cuprizone treatment induced demyelination.

Since in our study we looked at gain of function with the overexpression of PDGF-A, it would be interesting to examine the effect of a loss of PDGF-A function, or its cognate receptor, during chronic demyelination. Chronic demyelination to assess a loss of function of PDGF-A would have to be analyzed with either PDGF-A or PDGFαR conditional knock out mice. Conditional knock outs would have to be used to address this as it is impossible to do remyelination work in PDGF-A knock out mice. As the majority of PDGF-A knock out mice die by two weeks, and the rare ones that do survive do not live past six weeks and display a hypomyelinating phenotype with tremor and multiple developmental defects. Studies to address chronic demyelination also could not be analyzed with PDGFαR null mice, as the null phenotype is embryonic lethal.

However, chronic demyelination may be done in PDGFαR heterozygous mice, as these are viable, express half the normal protein levels of PDGFαR and previous studies have been done in these mice with acute cuprizone treatment (Murtie et al., 2005).

Further research to characterize the high and low affinity PDGFRs that bind PDGF-A are needed, as a low affinity receptor is believed to be associated with proliferation of oligodendrocyte lineage cells, while a 'novel' high affinity receptor is believed to be associated with survival (Barres et al., 1993). Currently only the PDGFαR and PDGFβR have been characterized, with the PDGFαR being the only one known to be expressed on OPs in the CNS. Determining the 'novel' receptor type that is associated with oligodendrocyte survival and the signaling cascade involved could lead to therapeutics that specifically activate this 'novel' receptor or components of its signaling cascade.

As the overexpression of PDGF-A influenced the survival of newly differentiated oligodendrocytes it would be prudent to examine if other growth factors known to positively effect oligodendrocyte survival are beneficial after chronic demyelination. Insulin and insulin growth factors (IGFs), neurotrophin-3 (NT3), ciliary-neurotrophic factor (CNTF), interleukin-6 (IL-6), and leukemia inhibitory factor (LIF) have been shown to influence oligodendrocyte survival *in vitro* (Barres et al., 1993). However, will the *in vitro* survival effects extend to oligodendrocytes generated after chronic demyelination *in vivo*? If these factors do in fact enhance oligodendrocyte survival after chronic demyelination the possibility of a growth factor 'cocktail' with low concentrations of each together may synergize to further enhance remyelination.

Taken together, the results of these studies have demonstrated that there are significant differences in the OP response to acute and chronic demyelination. With increased Myt1 expression in predominantly OPs as a common factor in demyelination in an effort to regenerate oligodendrocytes and enhance remyelination. While during chronic demyelination the regeneration of oligodendrocytes and concomitant remyelination does not necessitate the amplification of OPs to succeed, as has been postulated throughout the literature, but can be realized with growth factor based treatments, such as overexpression of PDGF-A, to enhance oligodendrocyte survival. Further work into the role Myt1 and growth factors may have after demyelination may bring about the design of potential therapeutics that offer profound benefits to promote remyelination of the CNS so important in a debilitating chronic disease such as MS.

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Appendix A

Abbreviations

BrdU – bromodeoxyuridine

cup - cuprizone

FGF – fibroblast growth factor

MHV – murine hepatitis virus strain-A59

Myt1 – myelin transcription factor 1

PBS – phosphate buffered saline

PDGF – platelet-derived growth factor

PDGFαR – platelet-derived growth factor alpha receptor

wpi – weeks post injection/infection